Supporting Information

Design, Synthesis, Molecular Docking, and Antimicrobial Evaluation of Novel Hybrid Peptides Derived from Unnatural Amino acids with Enhanced Hydrophobic Sidechains

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1. Synthesis and Characterization of Peptides 6a-e

1.1. Materials and Methods

The materials used in this study included 2-chlorotrityl chloride (2-CTC) resin, various Fmoc-protected *L*-amino acids, and reagents such as HBTU, HOBt·H_2O, DIPEA, TFA, and triisopropylsilane (TIS). The solvents employed were dichloromethane (DCM), dimethylformamide (DMF), methanol (MeOH), isopropyl alcohol (IPA), diisopropyl ether (DIPE), ethyl acetate, and n-hexane. These chemicals were sourced from reputable suppliers, including Merck, Sichuan, Survival Chemical, Spectrochem, and SD Fine Chemicals. The synthesis and characterization of peptides were carried out using equipment such as the CSBio peptide synthesizer (CS136X), a Bruker Avance 500 MHz spectrometer for NMR spectroscopy, Shimadzu high-performance liquid chromatography (HPLC) system for purity check, Preparative HPLC for purification of synthesized compounds, and Shimadzu LC-MS system for mass spectrometry.

1.2. Solid-Phase Peptide Synthesis

The solid-phase peptide synthesis protocol was employed to synthesize the novel peptides. The synthesis process began with the loading of Fmoc-Ala-OH on the 2-CTC resin. The resin was first washed with DCM and swelled to prepare it for the coupling reactions. Fmoc-Ala-OH was dissolved in DCM and introduced to the resin, along with DIPEA, to facilitate the coupling reaction. The mixture was stirred at 28 °C for two hours, ensuring the successful attachment of the cysteine derivative to the resin. Unreacted functional groups were capped using a solution of DIPEA, MeOH, and DCM, and the loading percentage was monitored using UV spectrophotometry.

The synthesis of peptides proceeded with iterative cycles of coupling and deprotection, characteristic of SPPS. The Fmoc group was deprotected using a 20% solution of piperidine in DMF. The resin was then washed with DMF and IPA to ensure thorough removal of the deprotection solution. The coupling reactions were performed using Fmoc-amino acids, HBTU, $HOBt \cdot H_2O$, and DIPEA in DMF. Each coupling step was monitored by the Kaiser test to confirm the completion of the reaction. This cycle of deprotection and coupling was repeated for each amino acid in the sequence, building up the peptide chain with the desired sequence.

Following the synthesis, the peptides were cleaved from the resin and deprotected using a cleavage cocktail composed of TIS, water, and TFA. The peptidyl resin was stirred in this cocktail at room temperature (RT) for three hours. The reaction mixture was then filtered, and the filtrate was precipitated using chilled DIPE. The mixture was stirred at low temperatures, filtered, and washed to yield the final peptides. These peptides were dried under vacuum to obtain them in powder form.

All the synthesized compounds were purified using preparative HPLC. Following are the HPLC details:

Mobile Phase A: 0.1 % TFA in Water
Mobile phase B: 0.1 %TFA in Acetonitrile
UV: 220 nm.
Crude Diluent: 10% Acetonitrile in Water
Loading Crude Qty. 3g (Each time)
Column: 50mm
Novasep column
Water system
Silica: YMC C18

Time	M-A	M-B	Flow rate
10	80 %	20 %	50 mL/min
45	65 %	35%	50 mL/min
60	50 %	50 %	50 mL/min
80	20 %	80 %	50 mL/min

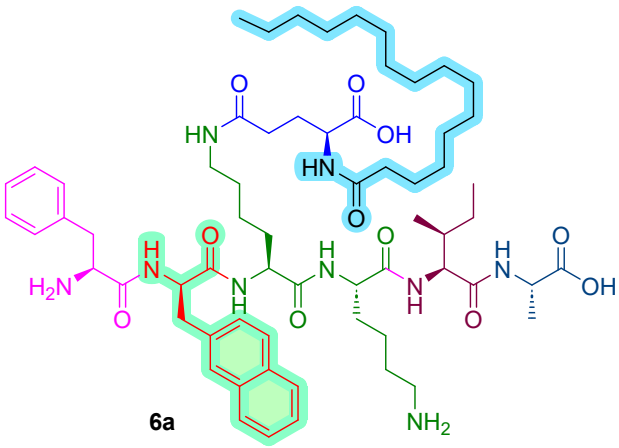
1.3. Characterization of Peptides 6a-e

Characterization of the synthesized peptides was crucial to confirm their structure and purity. NMR spectroscopy, including both ^{1}H NMR and ^{13}C NMR, was performed using a Bruker Avance 500 MHz spectrometer with DMSO-d_6 as the solvent.

Mass spectrometry was conducted using a Shimadzu LC-MS system in ESI mode to determine the molecular weights of the peptides. In the ESI mode, peptides often ionize to form doubly charged ions ($[M+2H]^{2+}$) due to the addition of two protons. As a result, the mass spectrometer detects a mass-to-charge ratio (m/z) that corresponds to half the actual molecular weight of each peptide, making the observed m/z value approximately half of the expected mass. Melting points of the peptides were measured using an open capillary method, and the purity of the peptides was confirmed through analytical HPLC. The HPLC purity check of all the compounds was done on Shimadzu HPLC system. The obtained purity of all the compounds was above 97%.

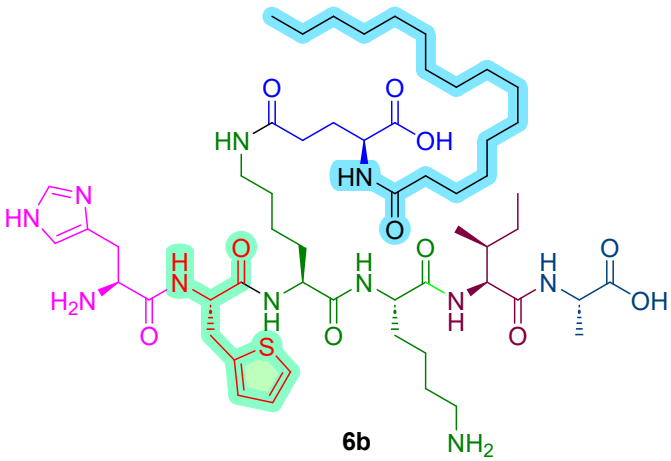
Melting points were measured using a Stuart SMP30 melting point apparatus with an open capillary method, providing data on the peptides' thermal properties.

3.3.1. D-Phe-D-Nal-Lys(Glu-Pal)-Lys-Ile-Ala (6a)



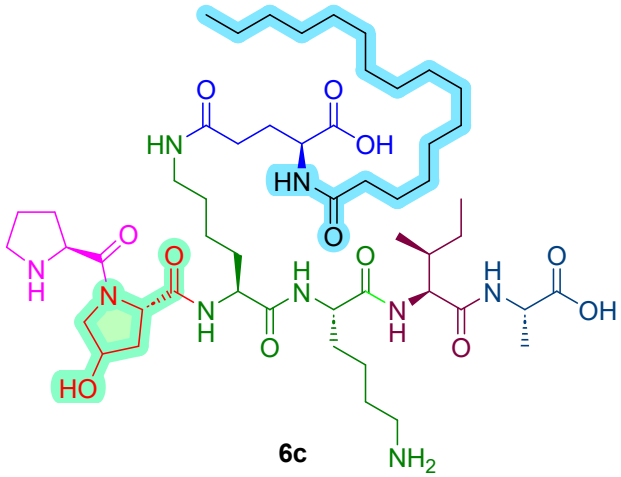
White cotton-like solid; **yield:** 86%; **purity:** 97%; **Optical rotation:** Observed rotation = -0.05°, $[\alpha]_D^{20.36}$ = -21.21° (c = 1.0, solvent: DMF); **M.P.:** 215 °C; **^{1}H NMR (500 MHz,** DMSO-d_6**)** δ 8.79 (d, *J* = 8.3 Hz, 1H), 8.33 (d, *J* = 8.1 Hz, 1H), 8.24 – 8.08 (m, 2H), 8.03 (d, *J* = 7.8 Hz, 1H), 7.82 (dt, *J* = 13.8, 7.1 Hz, 3H), 7.71 – 7.62 (m, 2H), 7.44 (q, *J* = 7.4 Hz, 3H), 7.31 – 7.14 (m, 5H), 4.83 (q, *J* = 7.8 Hz, 1H), 4.31 – 4.22 (m, 2H), 4.14 (dq, *J* = 14.9, 8.0 Hz, 4H), 4.03 – 3.94 (m, 2H), 3.12 (ddd, *J* = 15.6, 9.5, 4.1 Hz, 4H), 3.06 (d, *J* = 4.7 Hz, 1H), 3.00 (dd, *J* = 13.6, 8.2 Hz, 2H), 2.88 (q, *J* = 7.7 Hz, 2H), 2.81 (p, *J* = 6.9 Hz, 2H), 2.68 (t, *J* = 7.7 Hz, 2H), 2.08 (q, *J* = 8.3 Hz, 4H), 1.98 – 1.86 (m, 1H), 1.79 – 1.57 (m, 4H), 1.47 (tq, *J* = 15.2, 7.2 Hz, 7H), 1.20 (s, 20H), 1.05 – 0.91 (m, 3H), 0.82 (q, *J* = 6.4 Hz, 6H), 0.76 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR (126 MHz,** DMSO-d_6**)** δ 174.40, 174.00, 172.99, 171.91, 171.57, 171.54, 171.06, 170.45, 168.30, 135.23, 135.08, 133.40, 132.37, 130.01, 129.00, 128.23, 128.04, 128.01, 127.90, 127.83, 127.53, 126.43, 56.89, 54.45, 53.65, 52.87, 52.61, 51.96, 48.03, 35.53, 32.22, 31.71, 29.45, 29.43, 29.41, 29.37, 29.22, 29.20, 29.11, 29.04, 27.54, 27.12, 25.67, 24.41, 22.74, 22.51, 17.55, 15.59, 14.38, 11.45. **HRMS:** Chemical Formula: $C_{64}H_{99}N_9O_{11}$: Exact monoisotopic mass = 1169.7464. Calculated mass for [M+2H] = 1171.7621. HRMS (ESI, $[M+2H]^{2+}$): calculated m/z = 585.8805, observed m/z = 585.8853 (monoisotopic, error: 8.19 ppm).

3.3.2. His-Thi-Lys(Glu-Pal)-Lys-Ile-Ala (6b)



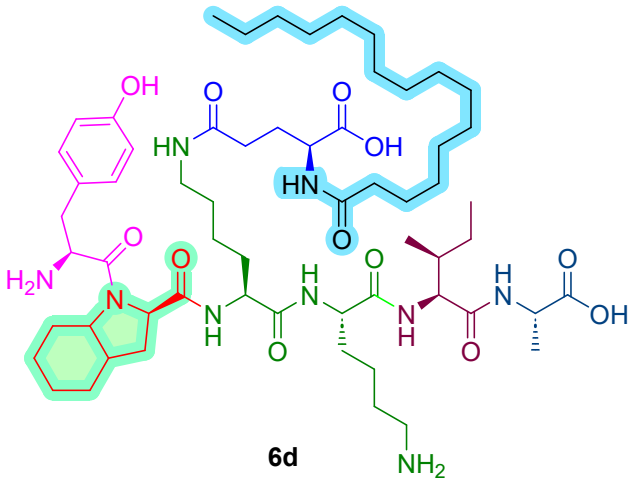
White powder; **yield:** 85%; **purity:** 99%; **Optical rotation:** Observed rotation = -0.05°, $[\alpha]_D^{19.76}$ = -20.04° (c = 1.0, solvent: methanol); **M.P.:** 198 °C; **^{1}H NMR (500 MHz,** DMSO-d_6**)** δ 8.83 (d, *J* = 7.7 Hz, 1H), 8.60 (d, *J* = 7.9 Hz, 1H), 8.24 (d, *J* = 7.0 Hz, 1H), 8.03 (dd, *J* = 7.8, 3.7 Hz, 2H), 7.81 (t, *J* = 5.6 Hz, 1H), 7.76 – 7.65 (m, 4H), 7.36 – 7.29 (m, 1H), 7.20 (s, 1H), 6.94 – 6.90 (m, 2H), 4.59 (ddd, *J* = 9.6, 7.7, 4.0 Hz, 1H), 4.27 (qd, *J* = 8.5, 5.3 Hz, 2H), 4.22 – 4.06 (m, 4H), 3.18 – 3.09 (m, 4H), 3.04 (dd, *J* = 15.1, 9.7 Hz, 2H), 2.95 (dq, *J* = 13.2, 6.7 Hz, 3H), 2.76 – 2.69 (m, 2H), 2.15 – 2.04 (m, 5H), 1.92 (dtd, *J* = 13.1, 7.9, 5.4 Hz, 1H), 1.79 – 1.57 (m, 4H), 1.56 – 1.38 (m, 8H), 1.26 – 1.18 (m, 33H), 1.05 (ddt, *J* = 16.4, 14.1, 7.4 Hz, 2H), 0.86 – 0.82 (m, 6H), 0.78 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR (126 MHz,** DMSO-d_6**)** δ 174.35, 174.00, 172.89, 171.79, 171.62, 171.49, 171.09, 170.82, 168.27, 158.77, 158.52, 139.68, 135.33, 127.28, 126.86, 125.13, 123.92, 56.80, 54.87, 53.22, 52.66, 51.90, 47.91, 37.49, 35.53, 32.21, 32.12, 31.73, 31.58, 29.49, 29.45, 29.41, 29.26, 29.14, 29.09, 27.55, 27.06, 25.69, 24.46, 23.24, 22.62, 22.53, 17.48, 15.60, 14.40, 11.45. **HRMS**: Chemical Formula: $C_{64}H_{99}N_9O_{11}S$: Exact monoisotopic mass = 1115.6777. Calculated mass for [M+2H] = 1117.6933. HRMS (ESI, $[M+2H]^{2+}$): calculated m/z = 558.8467, observed m/z = 558.8463 (monoisotopic, error: -0.63 ppm).

3.3.3. Pro-Hyp-Lys(Glu-Pal)-Lys-Ile-Ala (6c)



White cotton-like powder; **yield:** 86%; **purity:** 99%; **Optical rotation:** Observed rotation = -0.12°, $[\alpha]_D^{20.56}$ = -48.25° (c = 1.0, solvent: methanol); **M.P.:** 139 °C; **^{1}H NMR (500 MHz,** DMSO-d_6**)** δ 8.14 (dd, *J* = 9.9, 7.2 Hz, 2H), 7.99 (d, *J* = 7.8 Hz, 1H), 7.89 (d, *J* = 8.0 Hz, 1H), 7.77 (q, *J* = 4.2 Hz, 2H), 4.47 (dt, *J* = 15.3, 7.9 Hz, 1H), 4.33 (tt, *J* = 4.4, 2.1 Hz, 1H), 4.26 (td, *J* = 7.9, 6.2 Hz, 1H), 4.20 – 4.14 (m, 1H), 4.14 – 4.08 (m, 2H), 3.55 – 3.47 (m, 3H), 3.24 – 3.18 (m, 4H), 3.14 (dt, *J* = 11.2, 7.2 Hz, 3H), 2.97 (hept, *J* = 7.1 Hz, 3H), 2.72 (t, *J* = 7.4 Hz, 2H), 2.41 – 2.30 (m, 2H), 2.08 (t, *J* = 7.7 Hz, 4H), 1.93 – 1.69 (m, 6H), 1.60 (tt, *J* = 10.1, 4.9 Hz, 2H), 1.48 (dtd, *J* = 15.2, 8.1, 4.5 Hz, 6H), 1.40 – 1.31 (m, 2H), 1.22 (s, 30H), 1.12 – 0.99 (m, 2H), 0.88 – 0.74 (m, 9H). **^{13}C NMR (126 MHz,** DMSO-d_6**)** δ 174.46, 174.06, 172.80, 171.85, 171.62, 171.57, 171.26, 170.88, 167.14, 123.92, 69.38, 58.91, 57.00, 52.01, 48.24, 46.14, 37.34, 35.54, 32.23, 31.73, 29.50, 29.49, 29.45, 29.42, 29.36, 29.27, 29.15, 29.10, 27.16, 25.70, 24.46, 23.95, 22.60, 22.54, 17.75, 15.66, 14.40, 11.51. **HRMS:** Chemical Formula: $C_{52}H_{93}N_9O_{12}$; Exact monoisotopic mass = 1035.6944. Calculated mass for [M+2H] = 1037.7088. HRMS (ESI, $[M+2H]^{2+}$): calculated m/z = 518.8544, observed m/z = 518.8542 (monoisotopic, error: -0.39 ppm).

3.3.4. Tyr-Oic-Lys(Glu-Pal)-Lys-Ile-Ala (6d)

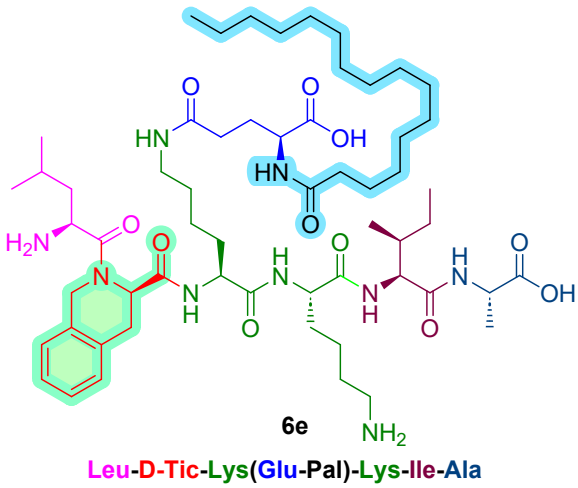


6d

Tyr-Oic-Lys(Glu-Pal)-Lys-Ile-Ala

White powder; **yield:** 79%; **purity:** 98%; **Optical rotation:** Observed rotation = -0.06°, $[\alpha]_D^{19.18}$ = -25.24° (c = 1.0, solvent: methanol); **M.P.:** 145 °C; **^{1}H NMR (500 MHz,** DMSO-d_6**)** δ 9.33 (s, 1H), 8.23 (d, *J* = 7.0 Hz, 1H), 8.09 – 8.00 (m, 2H), 7.98 (t, *J* = 7.7 Hz, 1H), 7.78 (t, *J* = 5.5 Hz, 1H), 7.73 (dd, *J* = 10.9, 8.9 Hz, 2H), 7.05 – 6.94 (m, 2H), 6.75 – 6.61 (m, 2H), 4.32 – 4.23 (m, 2H), 4.23 – 4.06 (m, 4H), 3.98 (td, *J* = 9.8, 4.6 Hz, 1H), 3.43 (d, *J* = 7.0 Hz, 1H), 2.96 (dq, *J* = 13.3, 6.3 Hz, 3H), 2.87 – 2.76 (m, 1H), 2.72 (q, *J* = 5.0 Hz, 2H), 2.09 (dtd, *J* = 10.4, 7.4, 3.3 Hz, 4H), 2.00 – 1.89 (m, 2H), 1.87 – 1.78 (m, 1H), 1.76 – 1.68 (m, 2H), 1.67 – 1.61 (m, 2H), 1.58 – 1.41 (m, 9H), 1.36 (qd, *J* = 10.5, 3.9 Hz, 5H), 1.28 – 1.18 (m, 32H), 1.16 – 1.00 (m, 3H), 0.87 – 0.81 (m, 6H), 0.78 (td, *J* = 7.4, 1.6 Hz, 3H). **^{13}C NMR (126 MHz,** DMSO-d_6**)** δ 174.37, 174.01, 172.84, 171.95, 171.87, 171.60, 171.51, 171.05, 170.94, 167.42, 158.30, 157.00, 131.15, 130.84, 115.85, 115.76, 60.07, 56.82, 52.61, 52.19, 51.95, 47.96, 39.15, 38.90, 37.50, 37.24, 35.53, 32.22, 31.74, 31.73, 29.50, 29.48, 29.45, 29.42, 29.27, 29.15, 29.09, 27.55, 27.12, 25.69, 24.46, 23.05, 22.62, 22.54, 17.55, 17.53, 15.63, 14.40, 11.48. **HRMS:** Chemical Formula: $C_{60}H_{101}N_9O_{12}$; Exact monoisotopic mass = 1139.7570. Calculated mass for [M+2H] = 1141.7710. HRMS (ESI, $[M+2H]^{2+}$): calculated m/z = 570.8785, observed m/z = 570.8867 (monoisotopic, error: 14.37 ppm).

3.3.5. Leu-D-Tic-Lys(Glu-Pal)-Lys-Ile-Ala (6e)



White powder; **yield:** 86%; **purity:** 98%; **Optical rotation:** Observed rotation = -0.05°, $[\alpha]_D^{20.44}$ = -19.47° (c = 1.0, solvent: methanol); **M.P.:** 164 °C**;** **^{1}H NMR (500 MHz,** DMSO-d_6**)** δ 8.19 (d, *J* = 8.2 Hz, 1H), 8.12 (d, *J* = 7.9 Hz, 1H), 8.07 (d, *J* = 8.3 Hz, 1H), 8.02 (dd, *J* = 7.8, 4.4 Hz, 2H), 7.98 (d, *J* = 7.9 Hz, 1H), 7.74 (dt, *J* = 10.4, 5.2 Hz, 3H), 7.23 – 7.10 (m, 6H), 4.94 (t, *J* = 5.5 Hz, 1H), 4.88 – 4.72 (m, 2H), 4.59 (d, *J* = 15.4 Hz, 1H), 4.53 – 4.46 (m, 1H), 4.21 (d, *J* = 7.0 Hz, 1H), 4.16 – 4.08 (m, 5H), 3.10 (d, *J* = 5.5 Hz, 2H), 3.03 – 2.80 (m, 4H), 2.70 (dt, *J* = 14.0, 7.5 Hz, 3H), 2.09 (dt, *J* = 10.6, 7.3 Hz, 6H), 2.00 – 1.86 (m, 2H), 1.73 (dd, *J* = 12.2, 5.3 Hz, 5H), 1.65 – 1.53 (m, 4H), 1.52 – 1.36 (m, 13H), 1.22 (d, *J* = 2.3 Hz, 35H), 0.96 (d, *J* = 6.4 Hz, 3H), 0.84 – 0.77 (m, 15H). **^{13}C NMR (126 MHz,** DMSO-d_6**)** δ 174.49, 174.04, 174.01, 172.85, 171.74, 171.61, 170.83, 170.34, 169.83, 158.65, 158.40, 133.60, 52.01, 49.42, 48.33, 37.37, 35.54, 32.25, 31.72, 29.48, 29.46, 29.43, 29.40, 29.25, 29.23, 29.13, 29.07, 27.63, 27.16, 25.68, 24.43, 23.91, 23.54, 23.46, 22.77, 22.53, 21.85, 21.31, 17.82, 17.73, 15.69, 15.65, 14.38, 11.54, 11.50. **HRMS:** Chemical Formula: $C_{58}H_{99}N_9O_{11}$; Exact monoisotopic mass = 1097.7464. Calculated mass for [M+2H] = 1099.7604. HRMS (ESI, $[M+2H]^{2+}$): calculated m/z = 549.8732, observed m/z = 549.8827 (monoisotopic, error: 17.25 ppm).

2. Computational Work

2.1. Experimental: Molecular Docking and Dynamics Study

This study investigated the potential inhibitory effects of synthesized peptides on the transglycosylase domain of the Penicillin-binding protein 1B (PBP1B) from *Escherichia coli* (PDB ID: 3VMA) through molecular docking and molecular dynamics (MD) simulations.

2.1.1. Peptide Preparation

The three-dimensional structures of the synthesized peptides were refined using the Glide LigPrep module in Schrödinger software.[1,2] This preparation involved optimizing the structures by performing energy minimization, correcting stereochemistry, and generating possible protonation states. LigPrep also generated multiple conformers to account for flexibility and potential binding conformations of each peptide, ensuring accurate input structures for docking.

2.1.2. Protein Preparation

The crystal structure of PBP1B was prepared using the Protein Preparation Wizard in Schrödinger, which involves several key steps to optimize the protein for molecular docking steps included:

(1) Adding missing atoms, loops, and side chains where needed.

(2) Correcting bond orders and assigning appropriate formal charges.

(3) Removing crystallographic water molecules that were not near the binding site.

(4) Performing an energy minimization to relieve any steric clashes or strain within the protein structure. This prepared protein structure was then used as the receptor in docking studies.

2.1.3. Docking Grid and Docking Procedure

A docking grid was generated around the active site of the transglycosylase domain using the Glide Receptor Grid Generation tool, which defined the boundaries of the binding site for accurate docking. Molecular docking was performed using the Standard Precision (SP) mode in Glide, which ranks ligands based on binding affinity and evaluates their interaction poses within the binding site of PBP1B.[3] The best binding conformations for each peptide were identified based on docking scores and interaction profiles.

2.1.4. Molecular Dynamics Simulation

To further analyze the stability and interaction dynamics of the docked complexes, molecular dynamics (MD) simulations were performed on the protein-ligand complexes using Schrödinger's Desmond application.[4] Key steps in the MD setup included:

System Solvation: Each protein-ligand complex was solvated in an SPC (simple point charge) water model, creating an orthorhombic simulation box with a 10 Å buffer around the complex.

Neutralization and Ionic Strength: Counter-ions were added to neutralize the system, and 0.15 M NaCl was introduced to mimic physiological ionic strength.

Energy Minimization and Equilibration: The systems were initially energy-minimized to remove any steric clashes. A subsequent heating phase brought the system to 300 K, followed by an equilibration phase to stabilize the temperature and pressure.

Production Run: A 100-nanosecond MD simulation was performed for each complex under constant temperature (300 K) and pressure (1 atm) in the NPT ensemble. The OPLS3e force field

was applied to model the molecular interactions within the system, providing an accurate depiction of both the protein and peptide behavior in a solvated environment.[5]

Throughout the simulation, data on key metrics such as RMSD, RMSF, and hydrogen-bond interactions were collected to assess the stability and binding characteristics of the peptides within the active site of PBP1B. The results from both molecular docking and MD simulations provide insights into the binding affinity and stability of the synthesized peptides as potential inhibitors of PBP1B.

2.2. Additional Figures

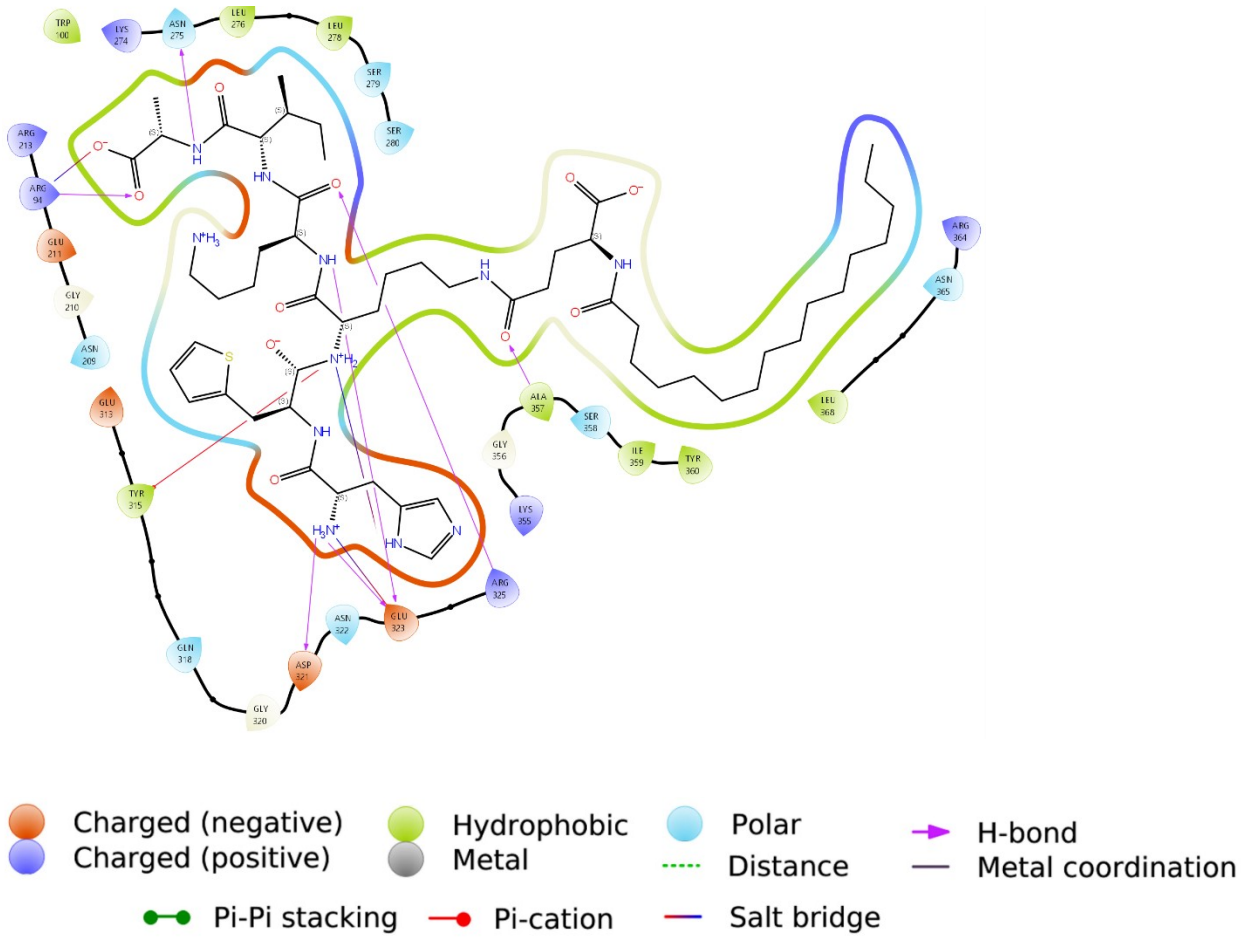


Figure S1. Interaction of **6b** with transglycosylase domain of PBP1b of *Escherichia coli*

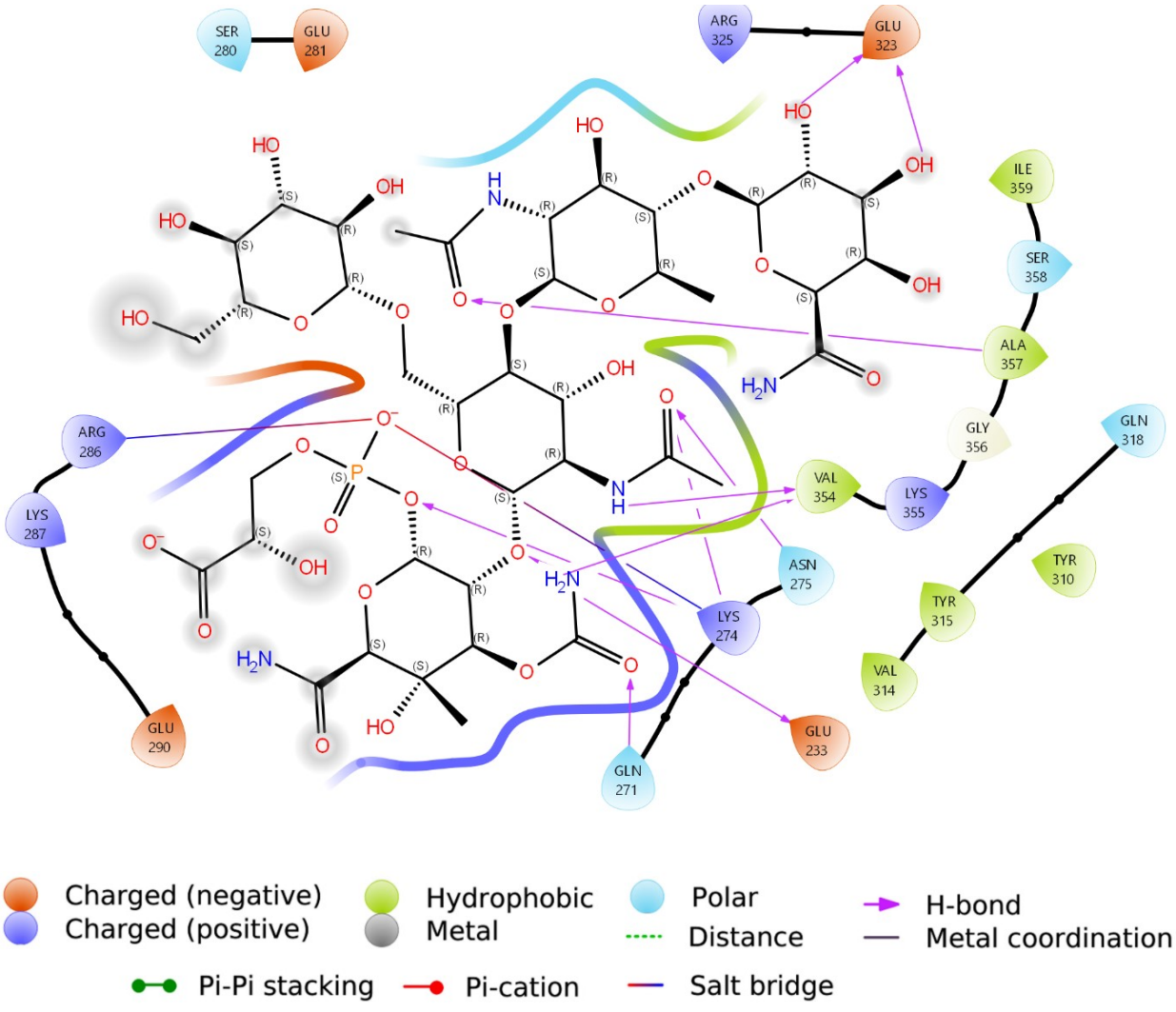


Figure S2. Interaction of Moenomycin with transglycosylase domain of PBP1b of *Escherichia coli*

3. Biological Assay

3.1. Experimental

The synthesized peptides **6a–e** were tested for their antimicrobial activity against a selection of bacterial and fungal strains.[6,7] The bacterial strains included *Escherichia coli* MTCC 443, *Bacillus subtilis* MTCC 121, *Bacillus megaterium* MTCC 428, and *Staphylococcus aureus* (MRSA) MTCC 96. Fungal strains included *Aspergillus niger* MTCC 282, *Aspergillus oryzae* MTCC 3107, *Rhizopus* spp. MTCC 262, and *Candida albicans* MTCC 227. All strains were obtained from the Microbial Type Culture Collection (MTCC), India, and stored at -70 °C in cryoprotectant-supplemented broth to maintain viability and purity until needed for assay.

3.1.1. Preparation of Media and Inoculum

The antibacterial activity of the peptides was assessed using the Mueller-Hinton Broth (MHB) microdilution method, following Clinical and Laboratory Standards Institute (CLSI) guidelines,[8] while Dextrose Broth (SDB) was used for antifungal testing.[9] Bacterial inocula were prepared by culturing each strain in MHB at 37 °C for 18–24 hours, adjusting the culture density to achieve a final concentration of approximately 10^8 CFU/mL, verified by optical density (OD) measurements. Fungal strains were cultured in SDB, adjusted to a concentration of 10^6 CFU/mL for yeast-like fungi (*C. albicans*) and 10^4 spore/mL for filamentous fungi (*A. niger*, *A. oryzae*, *Rhizopus* spp.).

3.1.2. Antibacterial and Antifungal Assay

For antibacterial assays, serial two-fold dilutions of each peptide were prepared in MHB to achieve concentrations ranging from 1000 µg/mL down to 1 µg/mL in 96-well microtiter plates. A standardized inoculum of each bacterial strain was added to each well, resulting in a final bacterial concentration of 5×10^5 CFU/mL. Plates were incubated at 37 °C for 24 hours, and the minimum

inhibitory concentration (MIC) was recorded as the lowest concentration of peptide that inhibited visible bacterial growth.[10]

In the antifungal assays, peptides were diluted in SDB for a concentration range of 1000 to 1 µg/mL. Each fungal strain was inoculated at a concentration of 1×10^4 spores/mL for filamentous fungi or 2×10^3 CFU/mL for *C. albicans*. Plates were incubated at 28 °C for 48 hours. MIC values were determined as the lowest peptide concentration that prevented visible fungal growth .

3.1.3. Controls and Standards

Ampicillin served as the reference antibacterial agent in the bacterial assays, while fluconazole was used in the fungal assays. Positive growth controls (containing no peptides) and sterility controls (containing only media) were included in each plate to validate results and ensure assay accuracy.

3.1.4. Data Collection and Analysis

All assays were conducted in triplicate, and the MIC values were reported as the mode of the observed values across replicates to enhance reliability. Results are presented as mean MIC values with standard deviations. The detailed data analysis aimed to determine the comparative efficacy of peptides **6a–e** relative to standard antibiotics .

3.2 Comparative Visualization of Activities

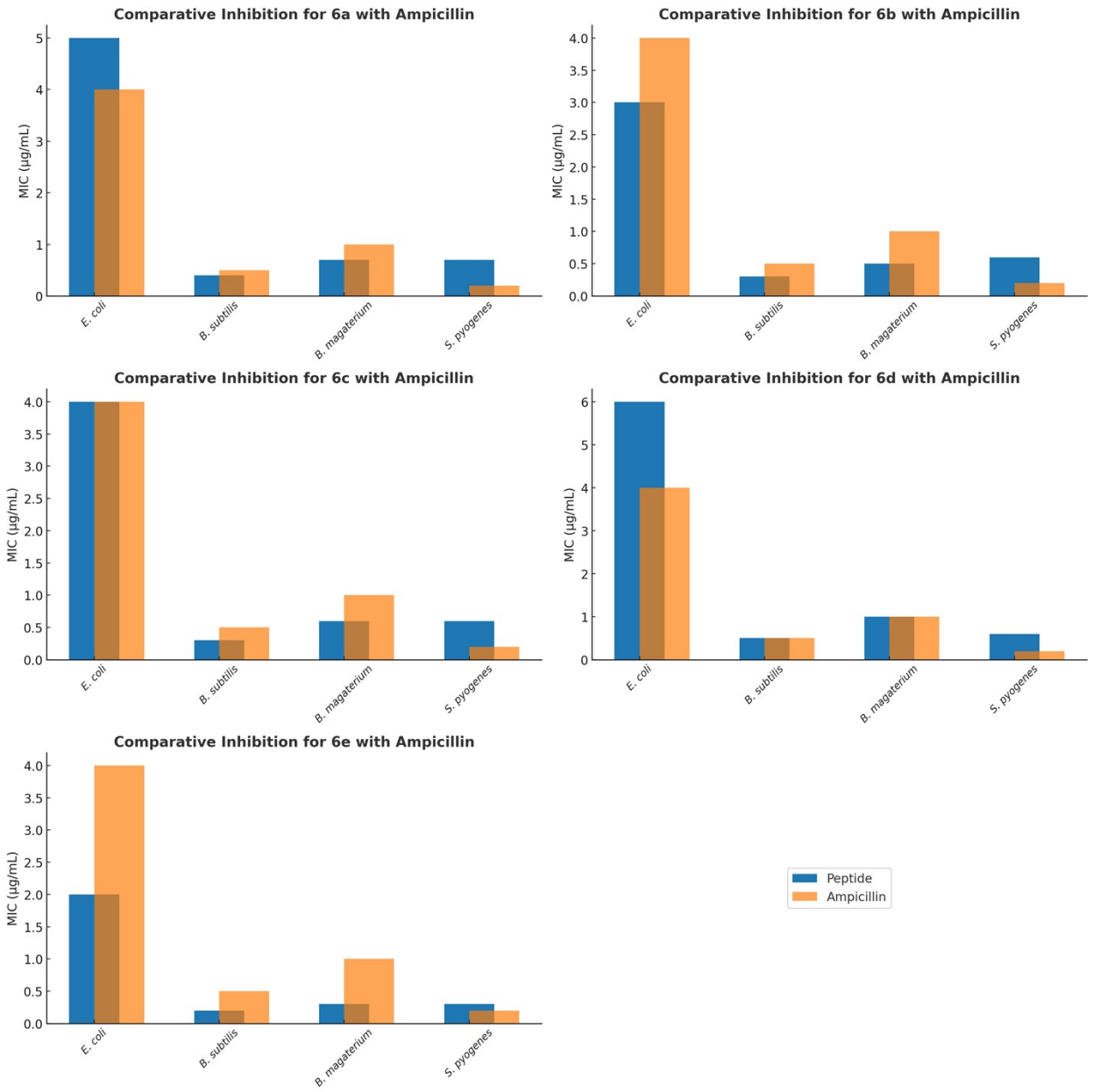


Figure S3. Antibacterial Activity Comparison: MIC values of peptides **6a-e** in comparison to Ampicillin against four bacterial strains: *E. coli, B. subtilis, B. magaterium*, and *S. pyogenes*. Each plot highlights the relative efficacy of each peptide in inhibiting bacterial growth in comparison to the standard antibiotic, Ampicillin.

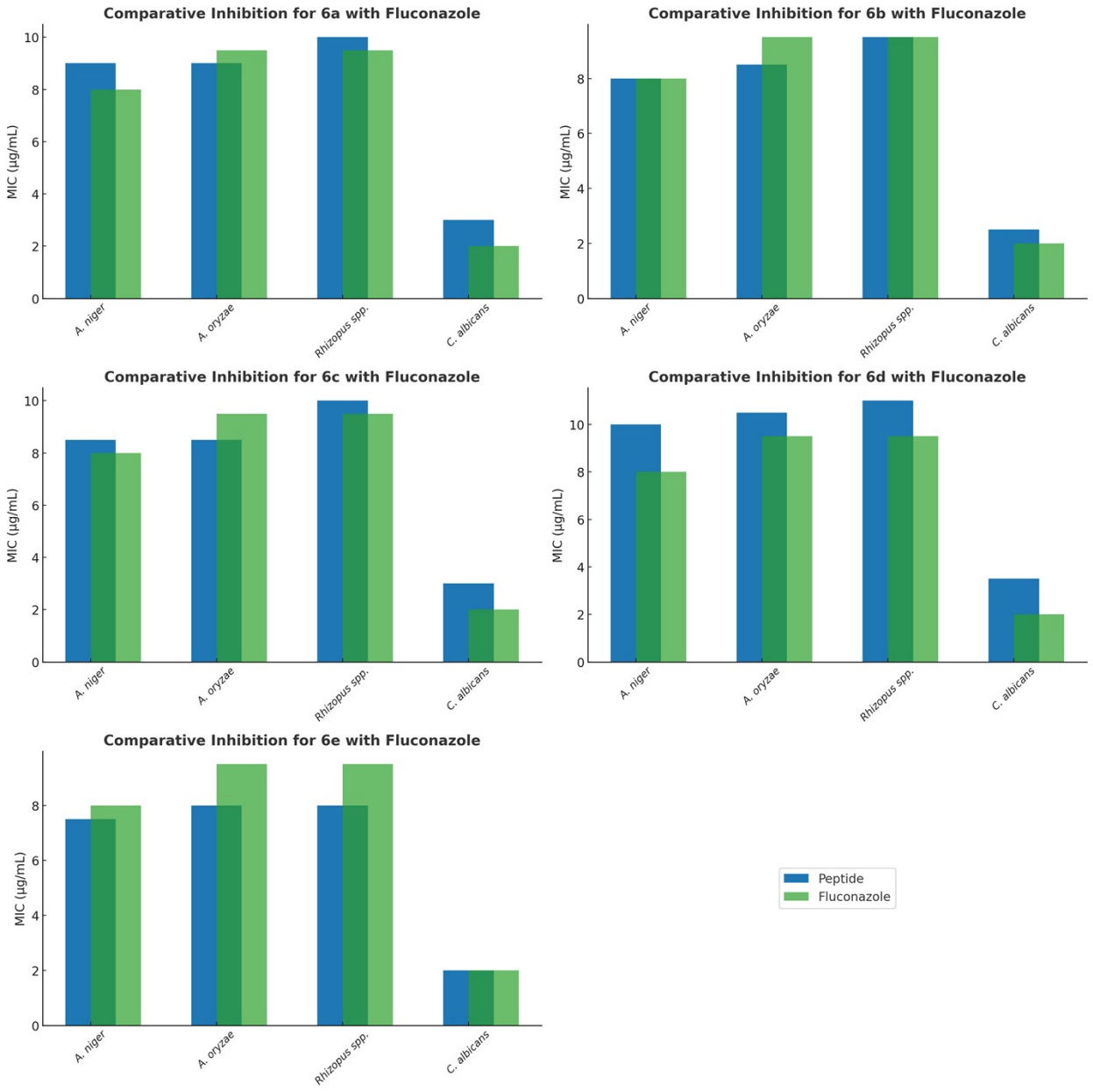


Figure S4. Antifungal Activity Comparison: MIC values of peptides **6a-e** in comparison to Fluconazole across four fungal strains: *A. niger, A. oryzae, Rhizopus spp.,* and *C. albicans*. Each subplot provides a comparative view of each peptide's antifungal activity relative to the standard antifungal agent, Fluconazole.

NMR and Mass Spectra

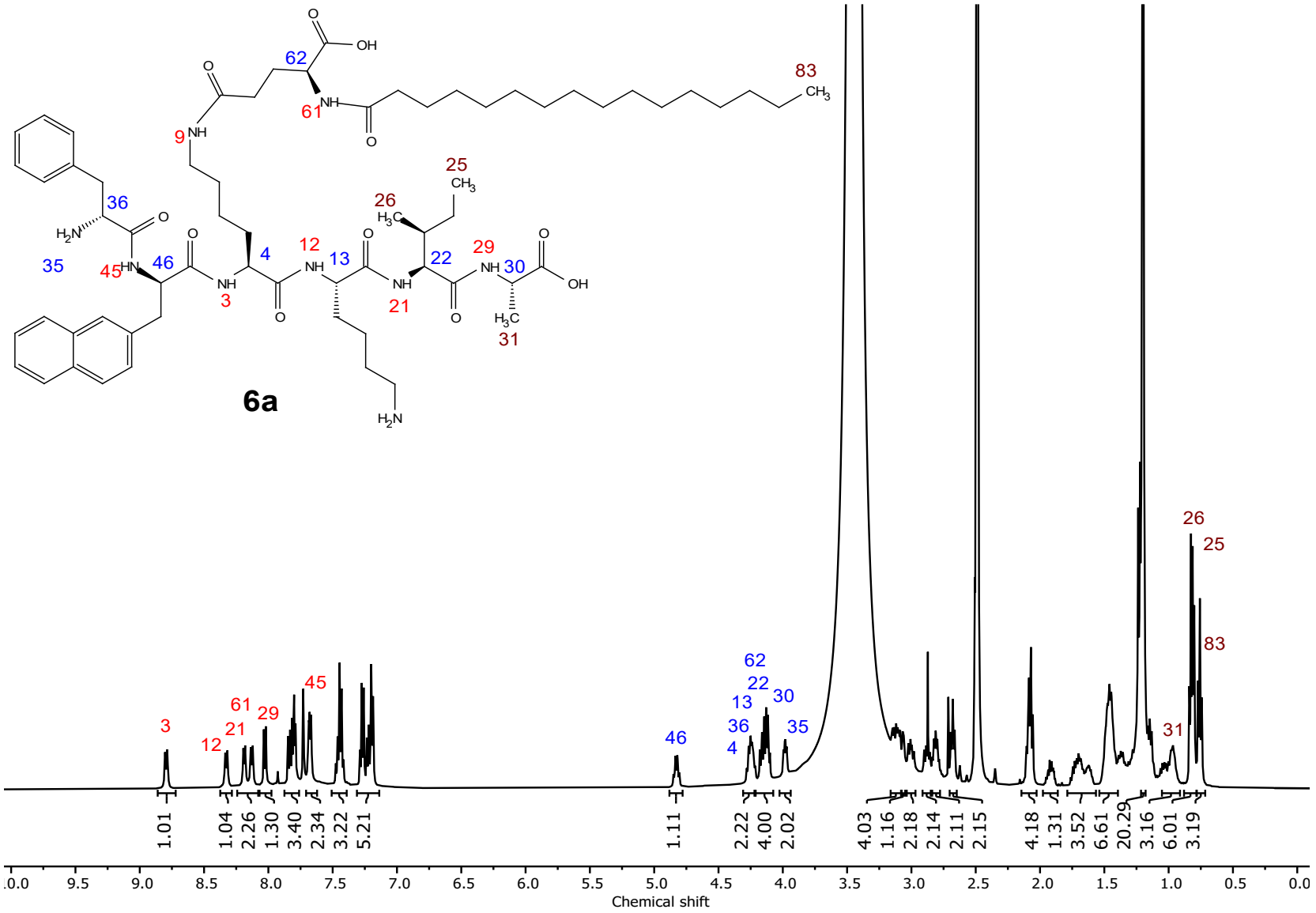


Figure S5. [1]H NMR spectrum of compound **6a**.

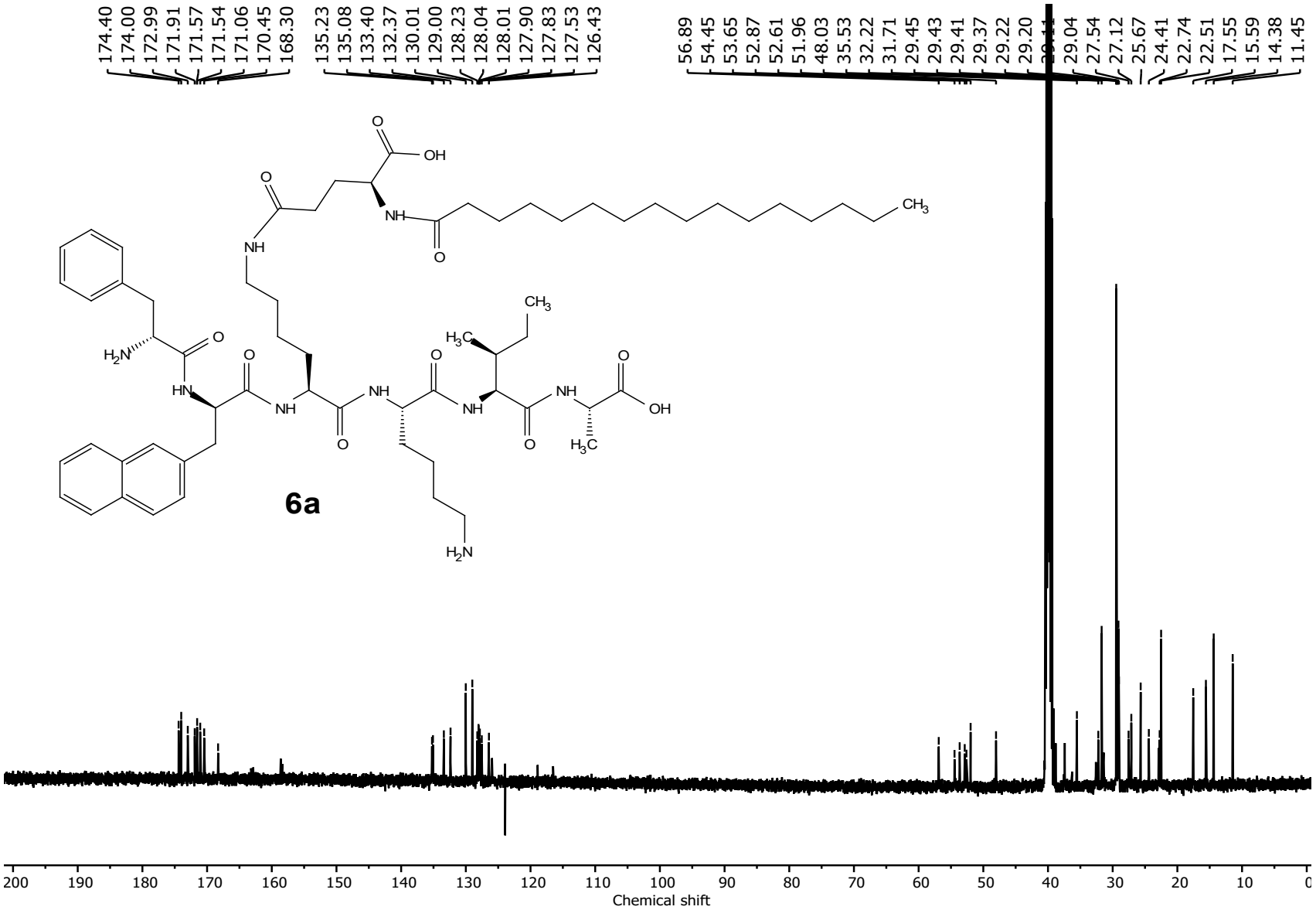


Figure S6. ^{13}C NMR spectrum of compound **6a**.

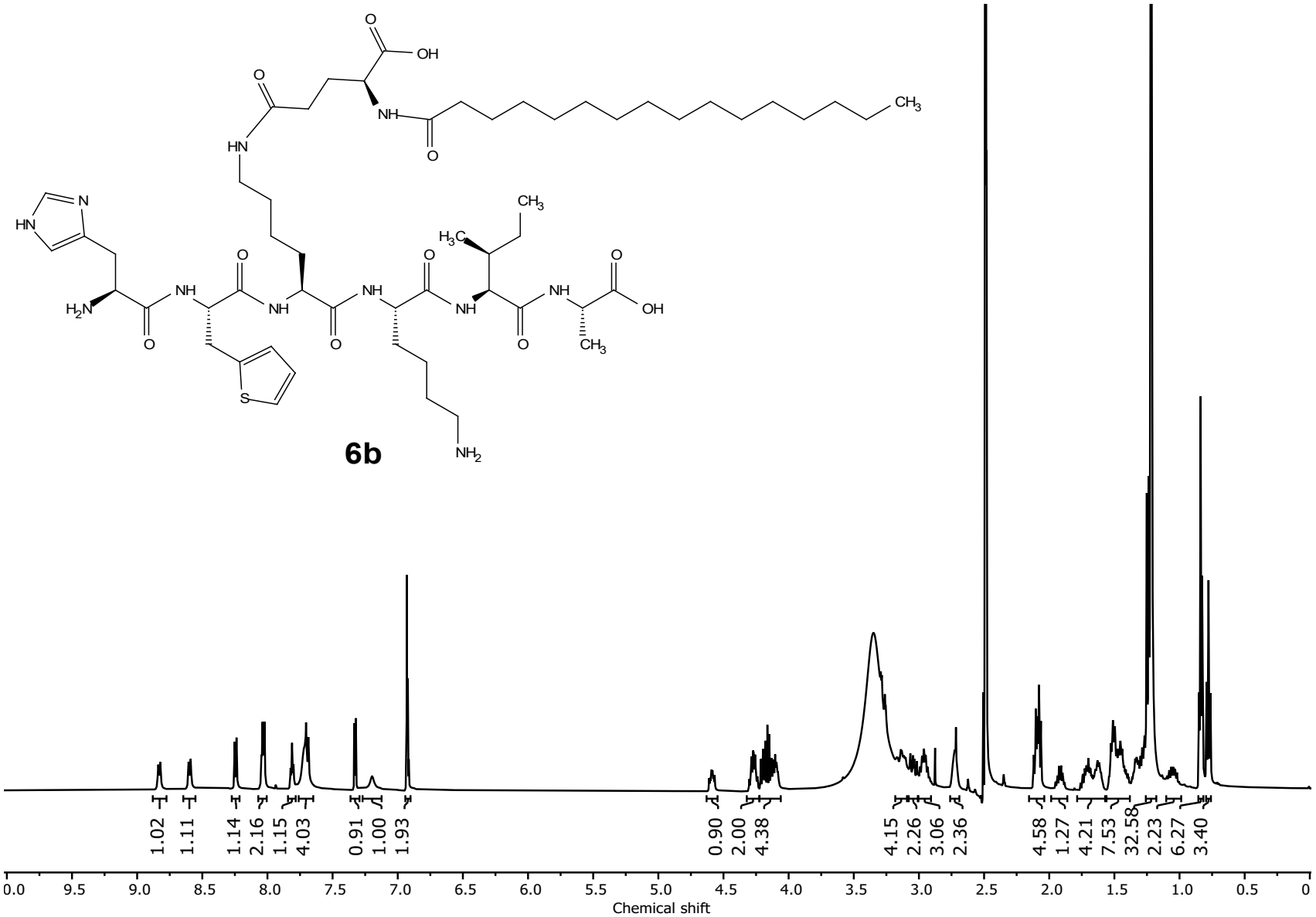


Figure S7. ^{1}H NMR spectrum of compound **6b**.

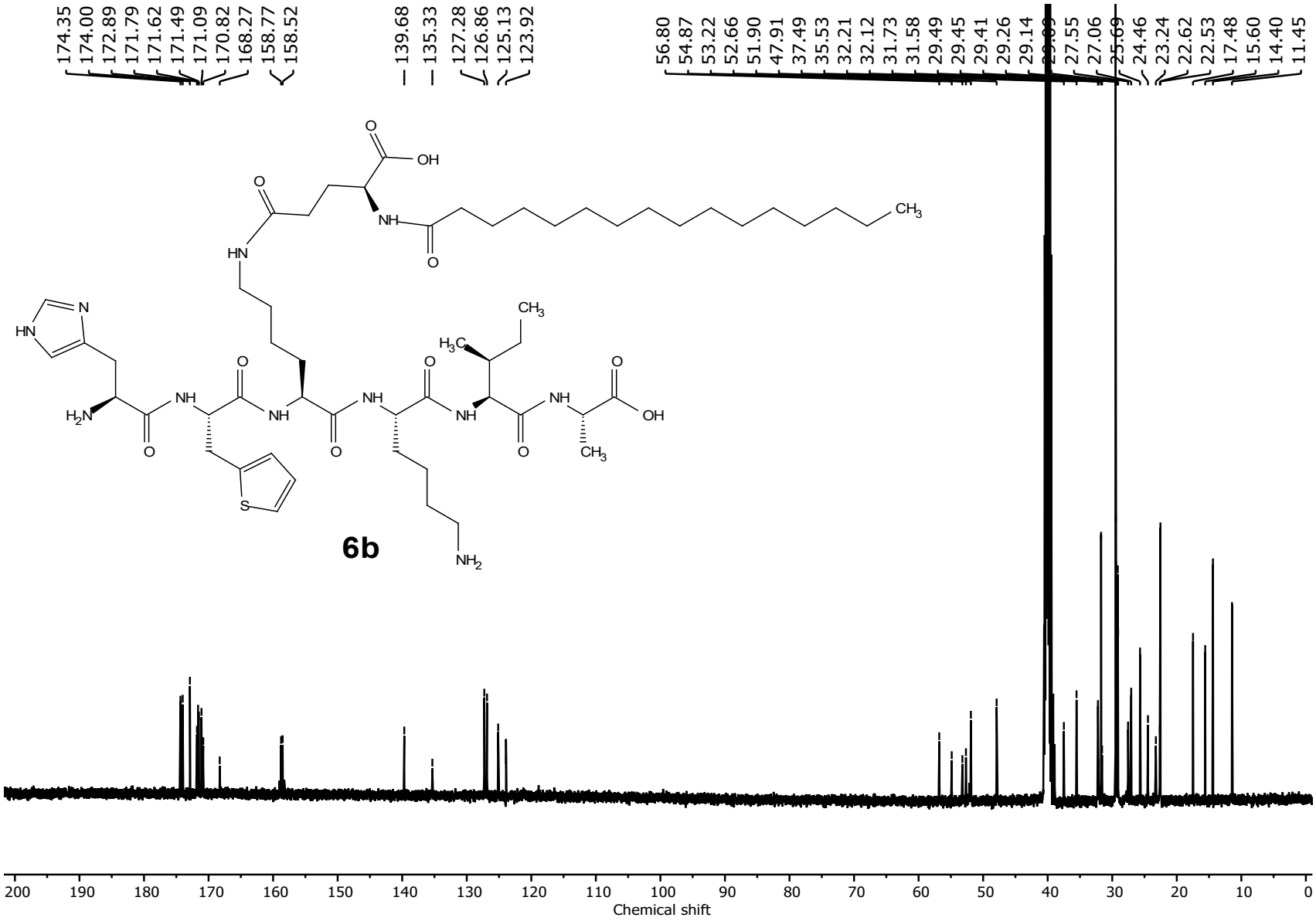


Figure S8. ^{13}C NMR spectrum of compound **6b**.

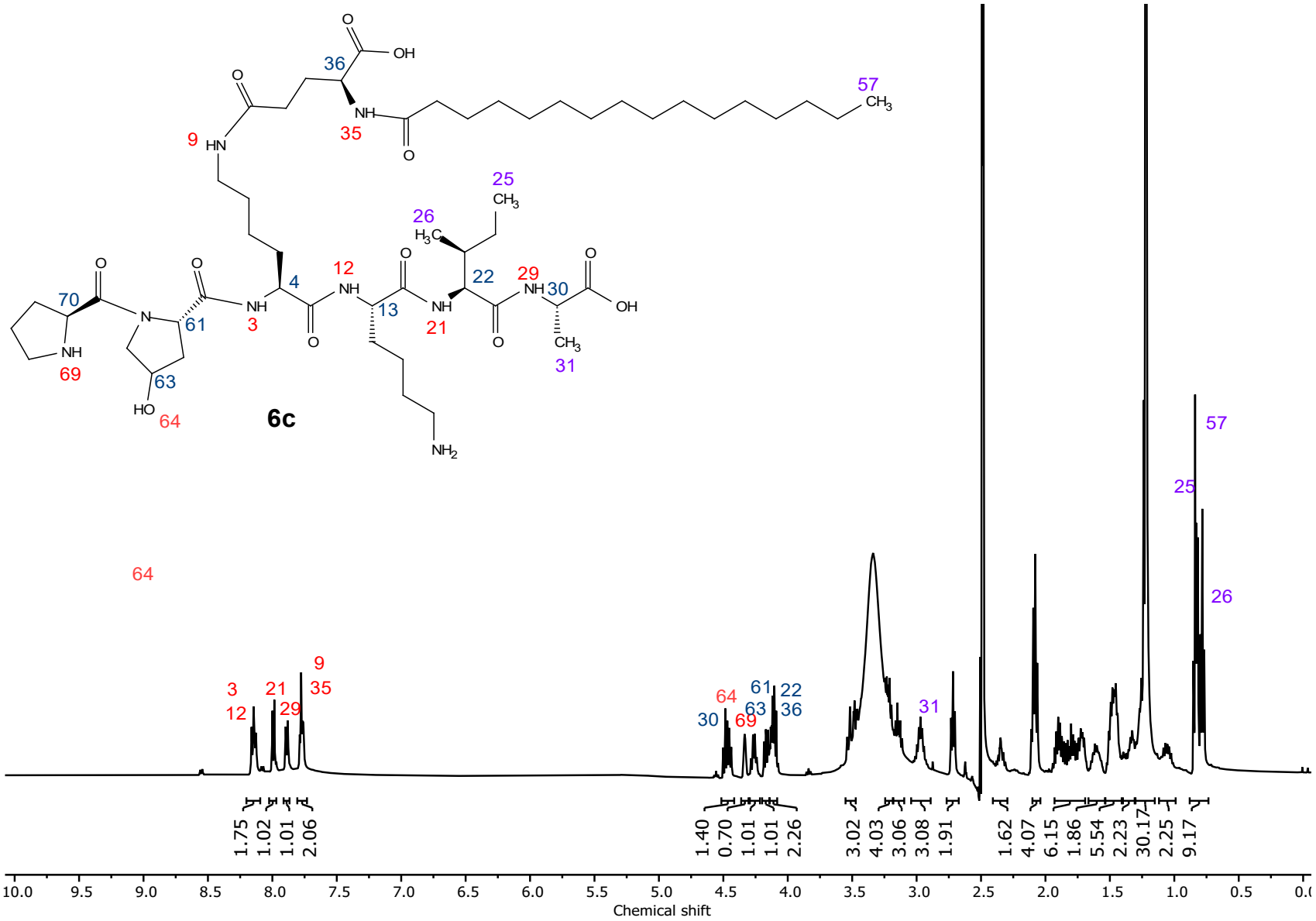


Figure S9. [1]H NMR spectrum of compound **6c**.

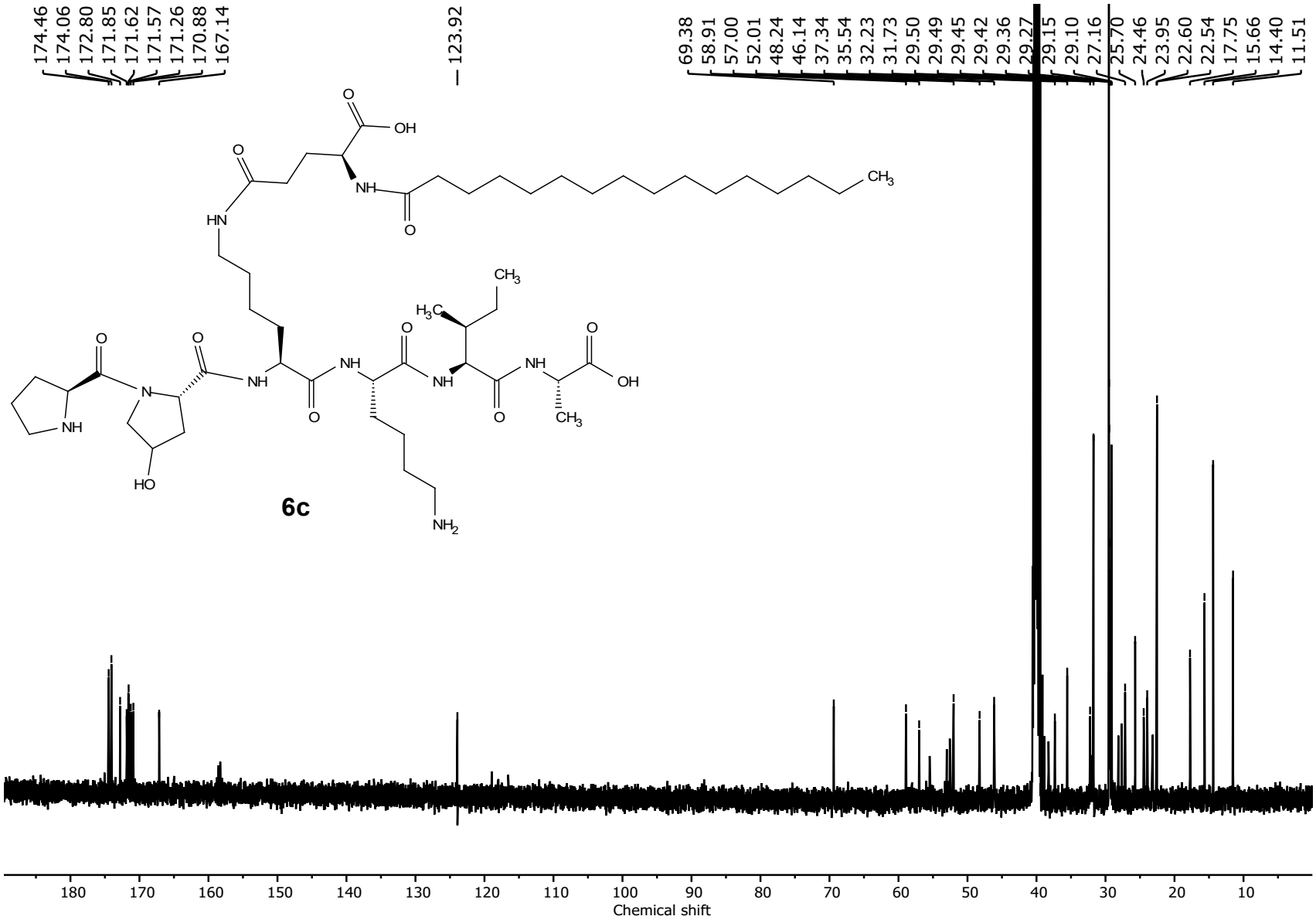


Figure S10. ^{13}C NMR spectrum of compound **6c**.

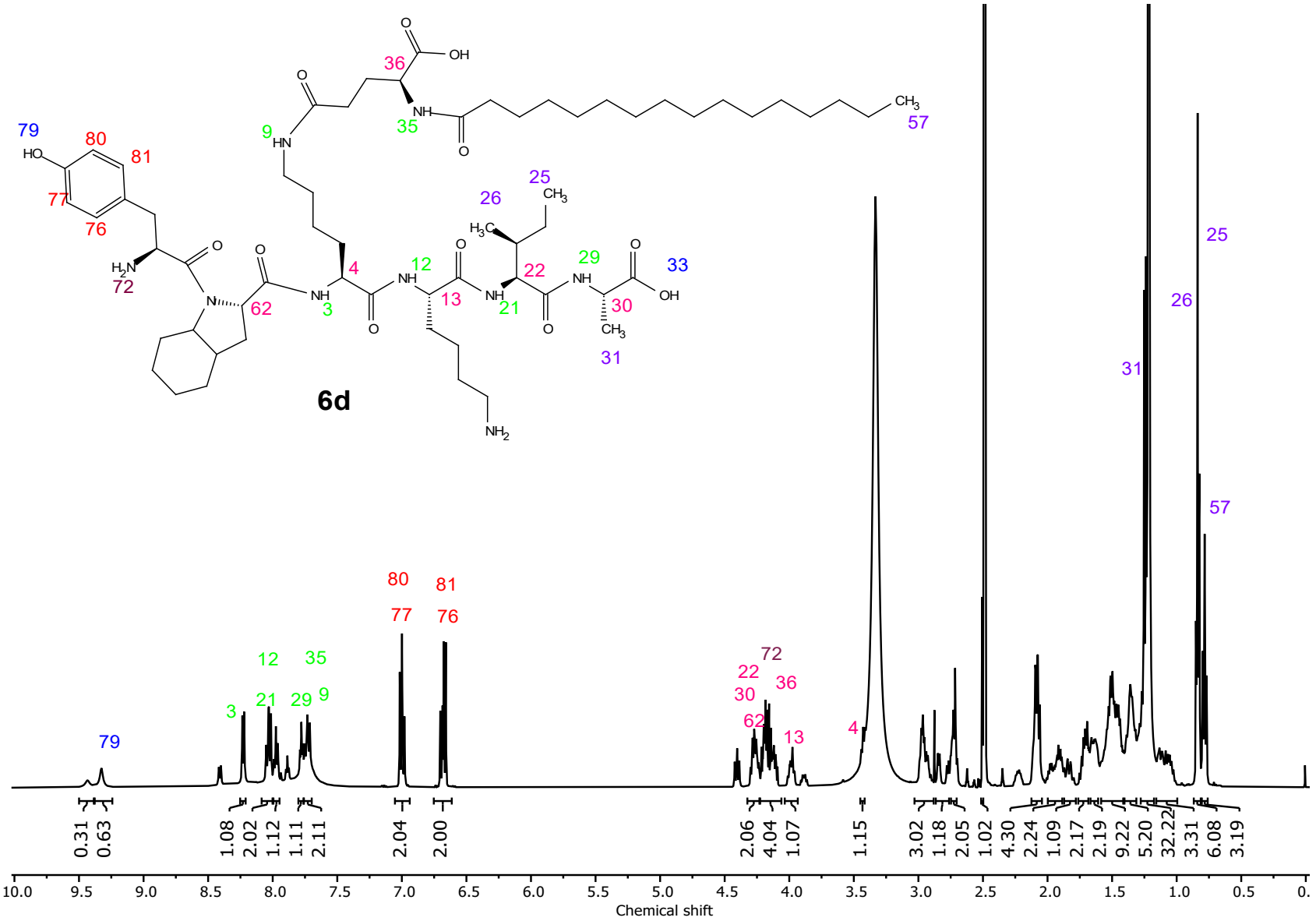


Figure S11. ^{1}H NMR spectrum of compound **6d**.

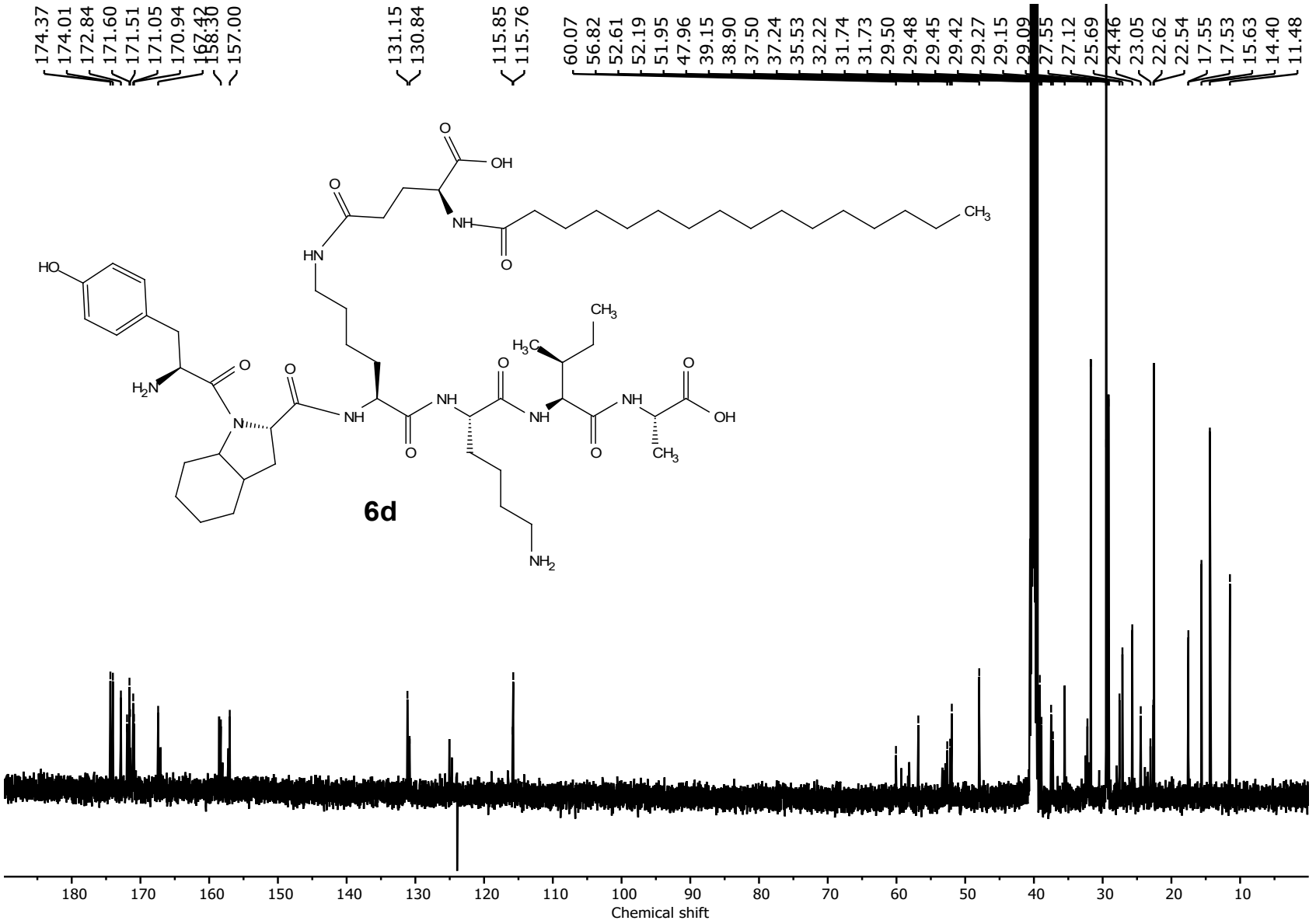


Figure S12. ^{13}C NMR spectrum of compound **6d**.

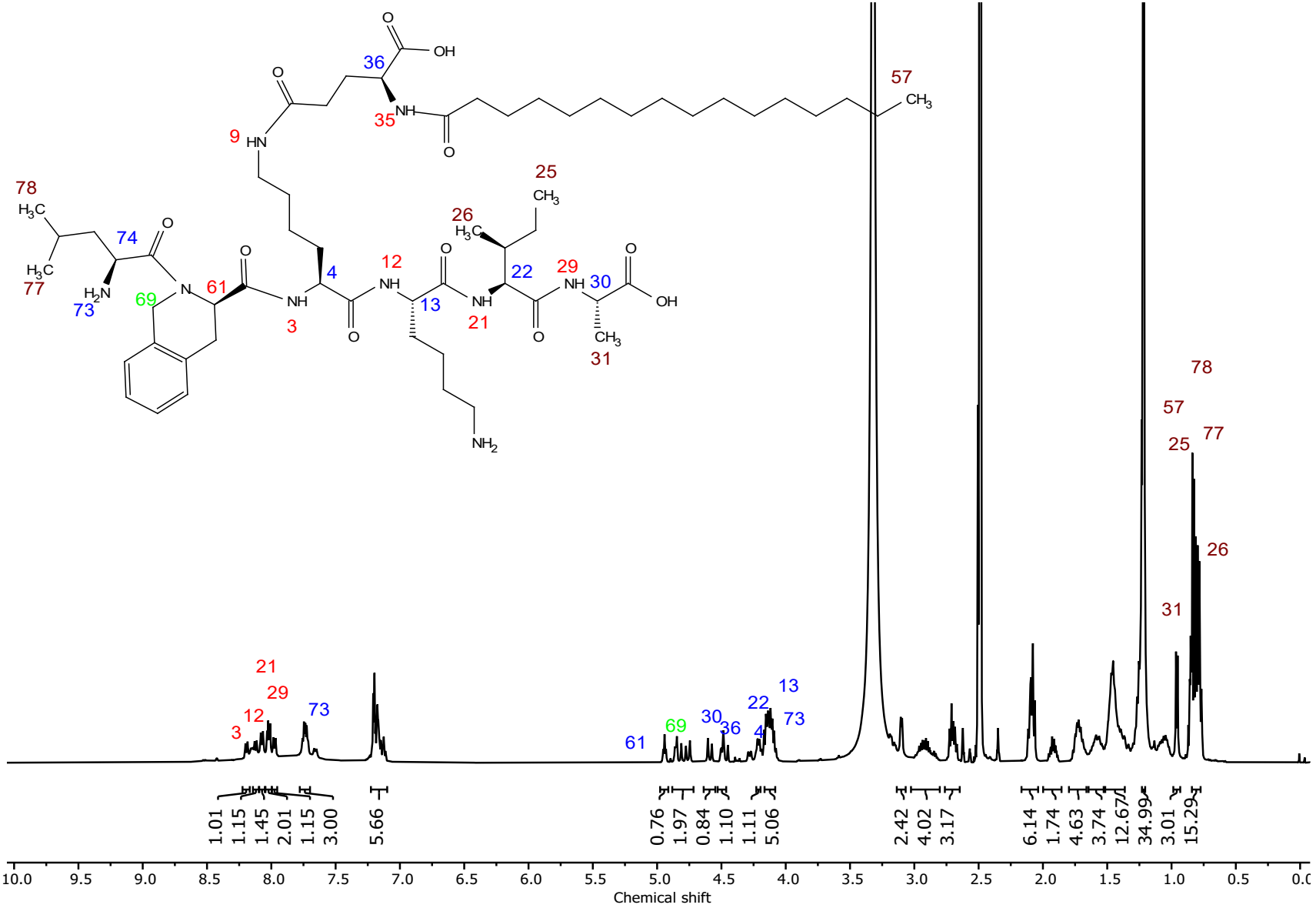


Figure S13. ^{1}H NMR spectrum of compound **6e**.

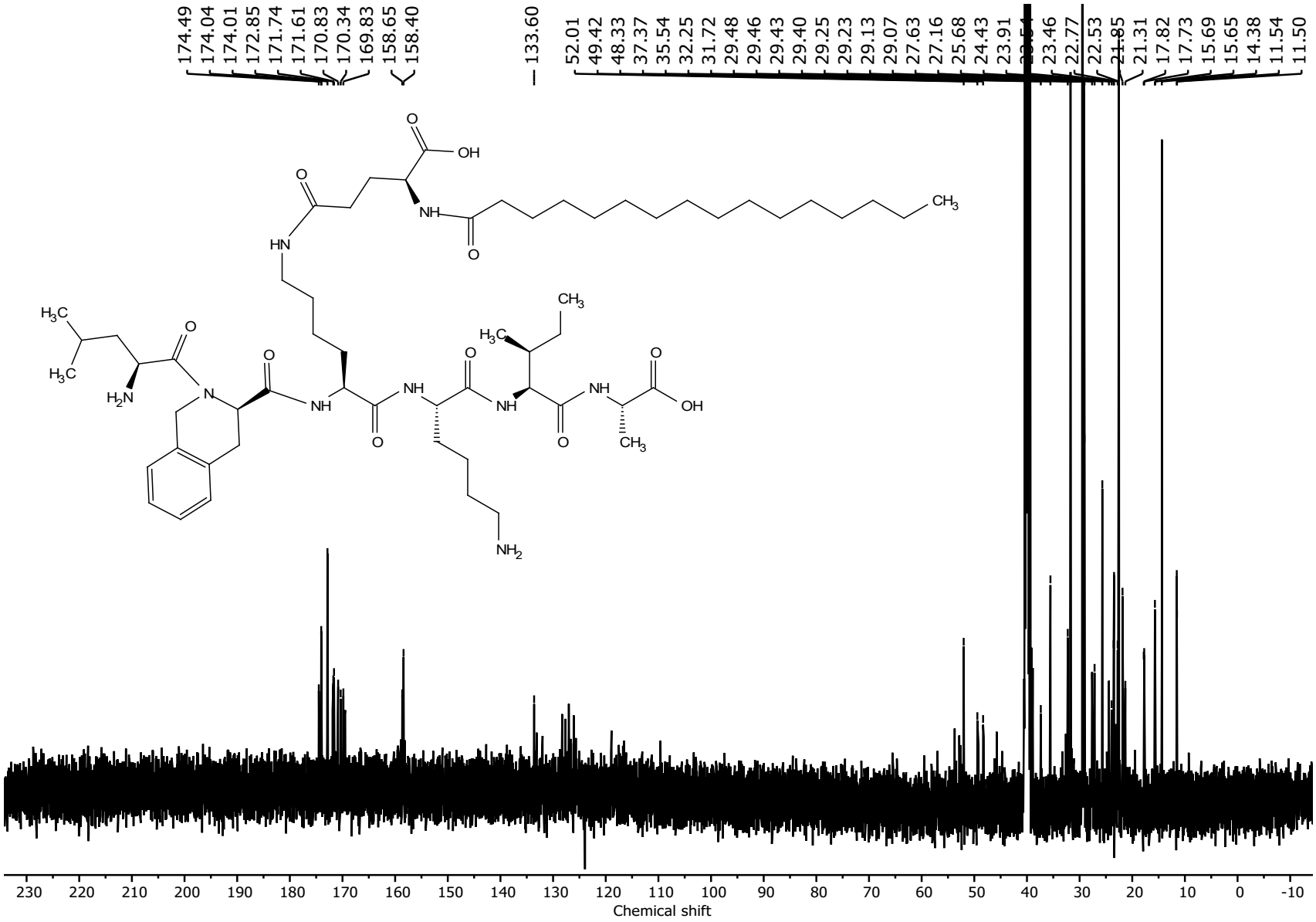


Figure S14. ^{13}C NMR spectrum of compound **6e**.

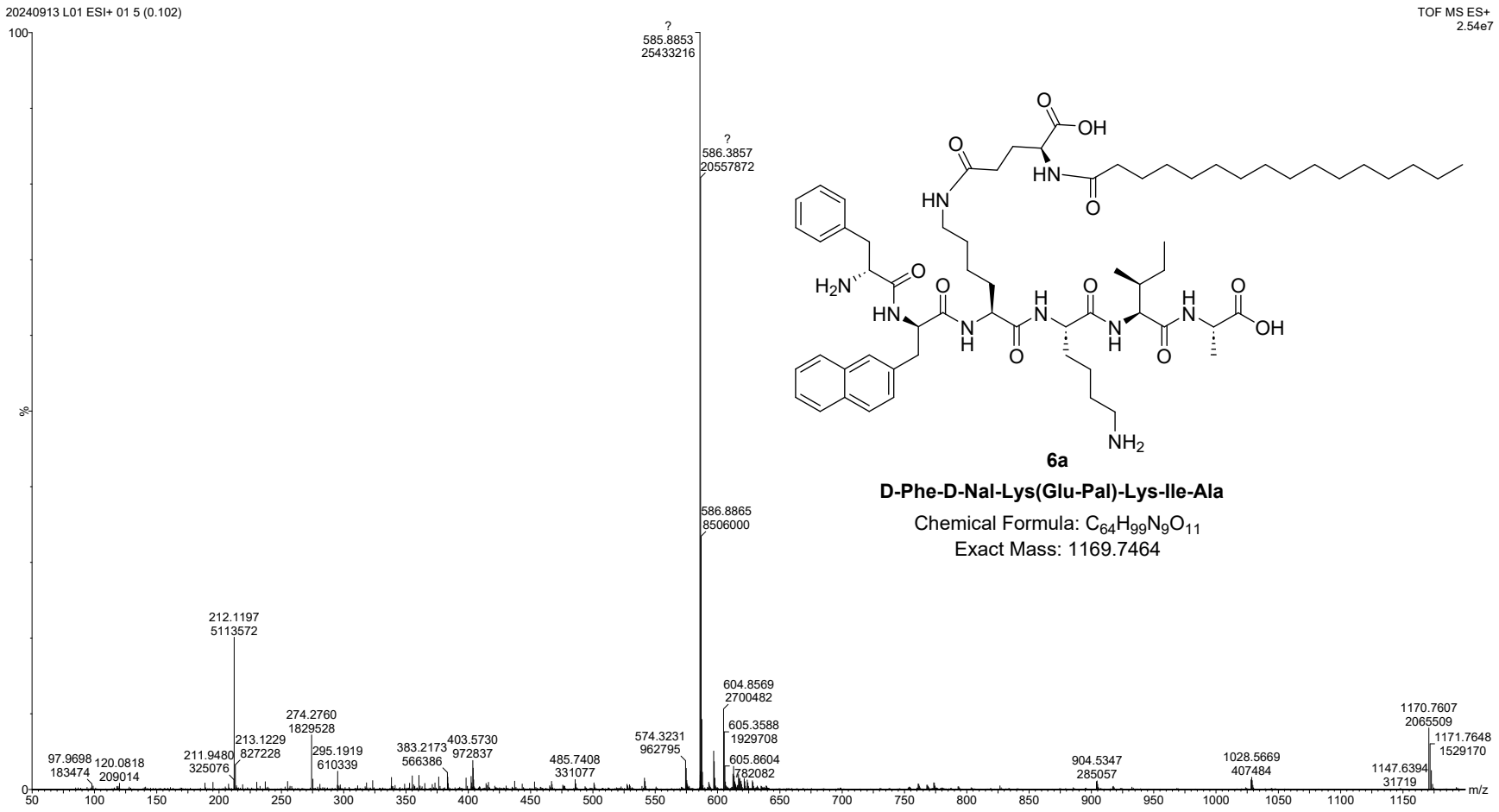


Figure S15. Mass spectrum of compound **6a**.

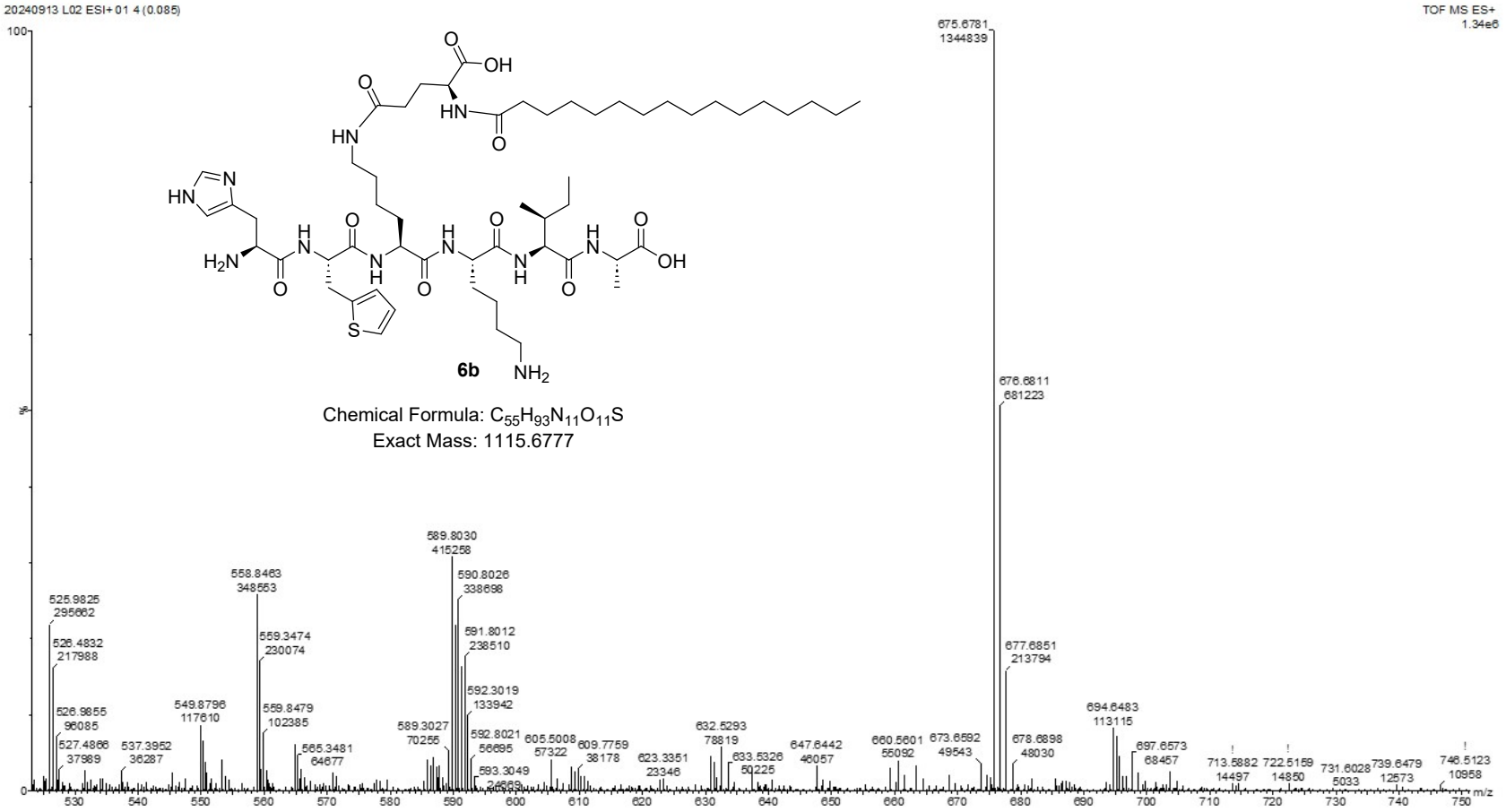


Figure S16. Mass spectrum of compound **6b**.

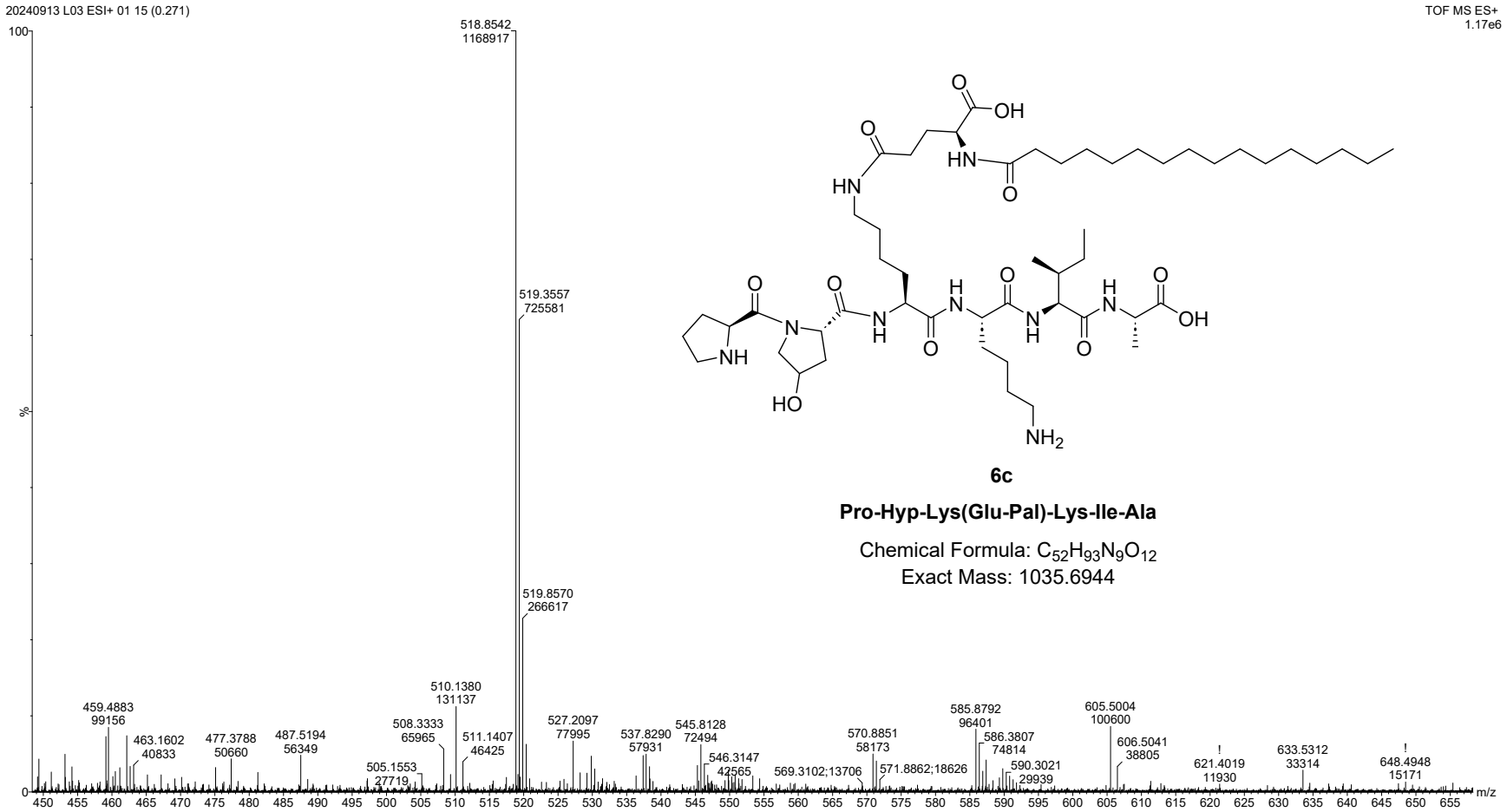


Figure S17. Mass spectrum of compound **6c**.

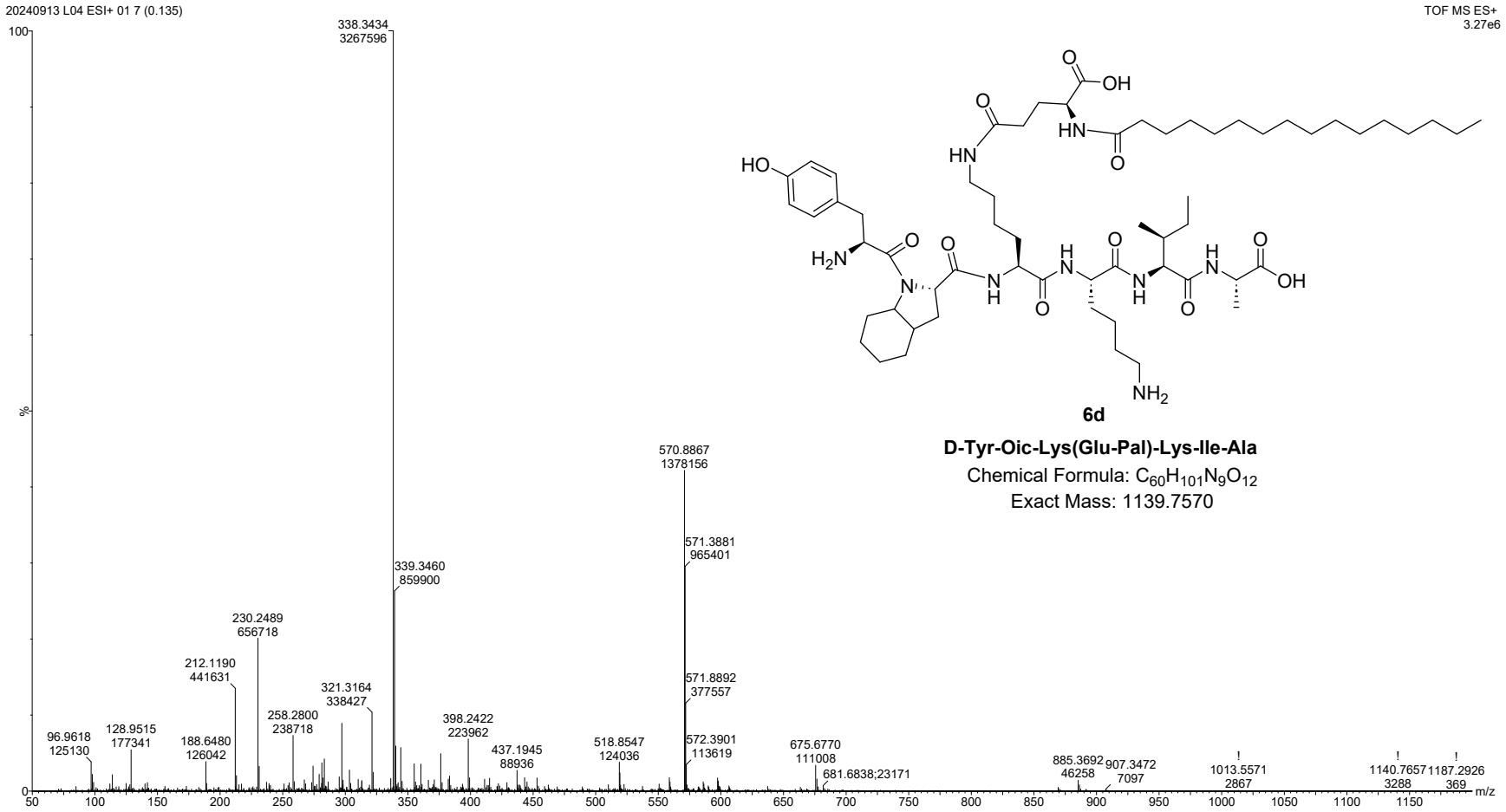


Figure S18. Mass spectrum of compound **6d**.

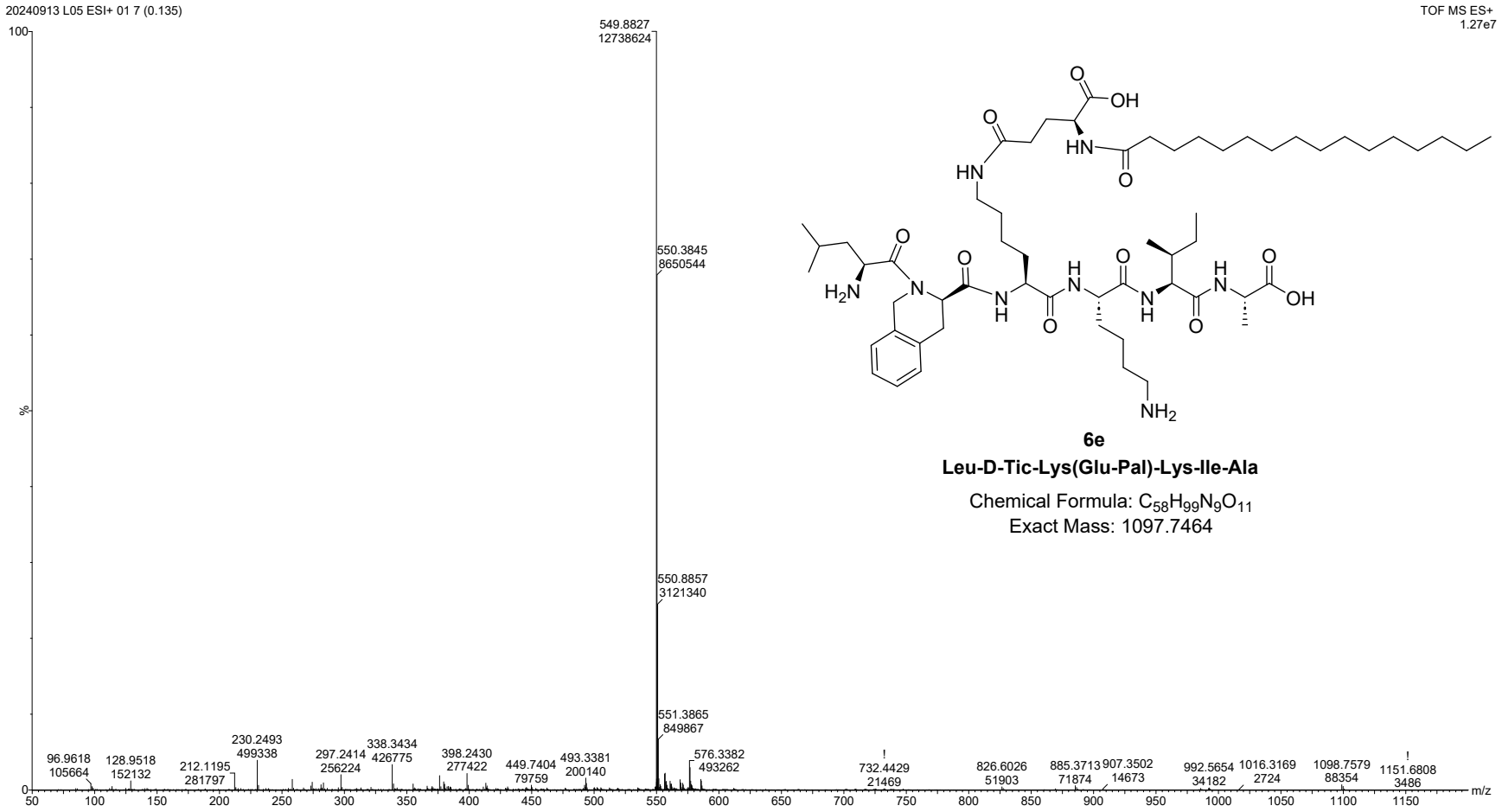


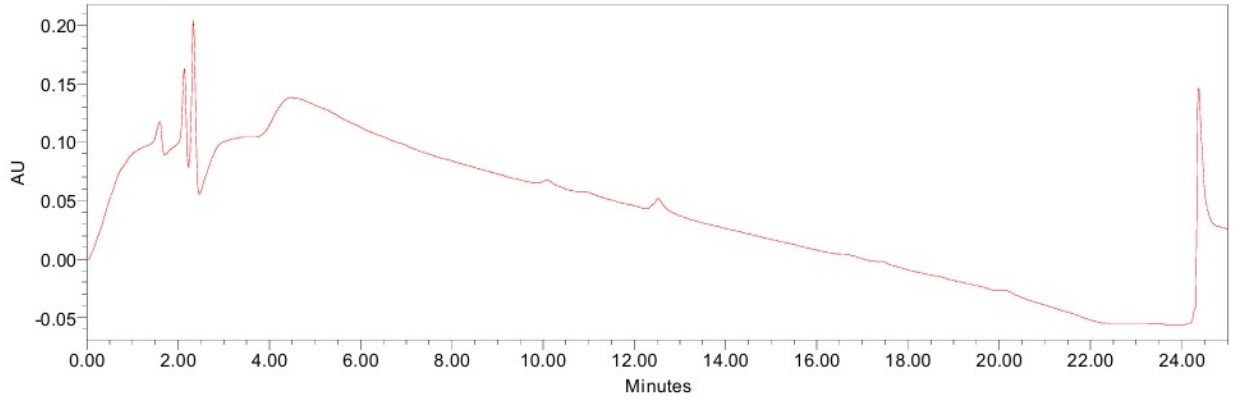
Figure S19. Mass spectrum of compound **6e**.

SAMPLE INFORMATION

Sample Name:	Blank	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	88	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	Blank
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 15:32:09 IST		
Date Processed:	14-11-2025 16:39:59 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1					

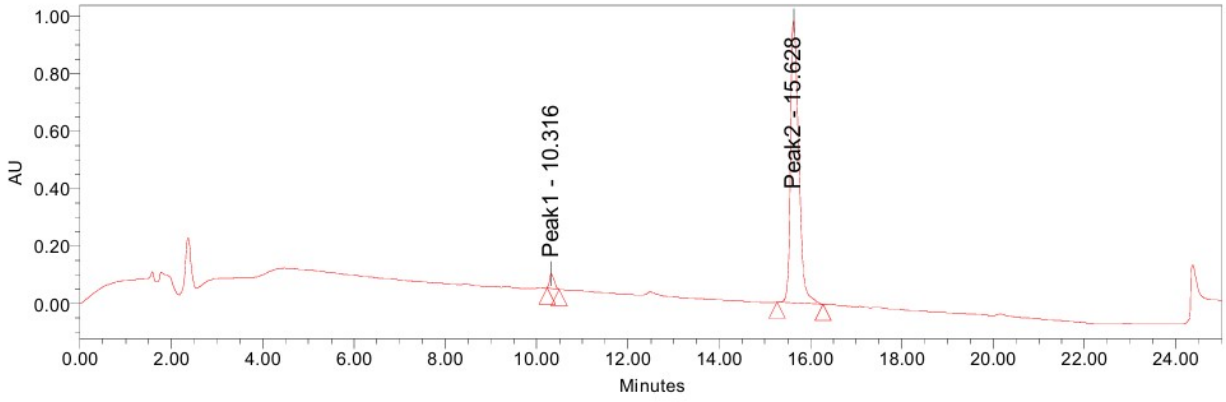
Figure S20. HPLC spectrum of Blank.

SAMPLE INFORMATION

Sample Name:	TIRZ/25-39- 8 MER ON-A	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	83	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	8 Mer ON_A
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 12:56:18 IST		
Date Processed:	14-11-2025 16:27:29 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1	Peak1	10.316	371272	51529	2.96
2	Peak2	15.628	12188515	982444	97.04

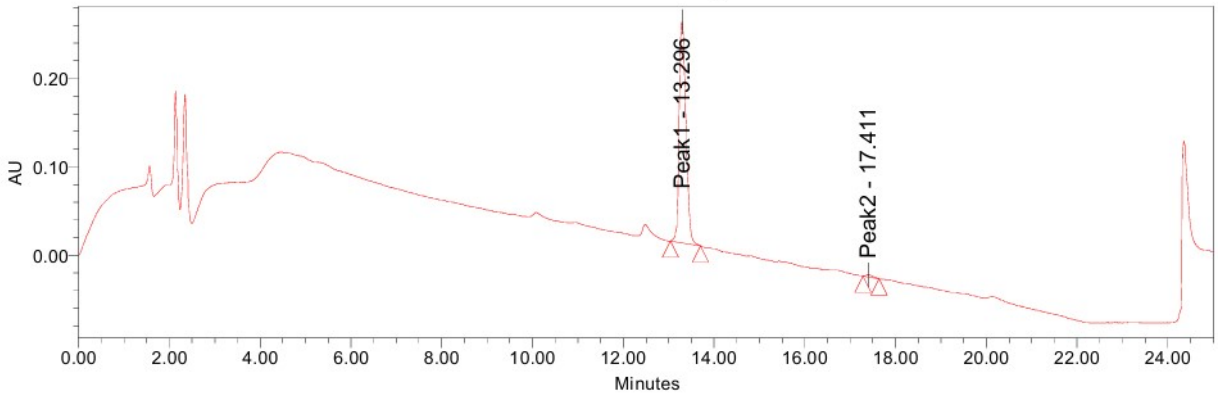
Figure S21. HPLC spectrum of Compound **6a**.

SAMPLE INFORMATION

Sample Name:	TIRZ/25-39- 8 MER ON-B	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	84	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	8 Mer ON_B
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 13:22:22 IST		
Date Processed:	14-11-2025 16:30:31 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5

Auto-Scaled Chromatogram



Peak Results

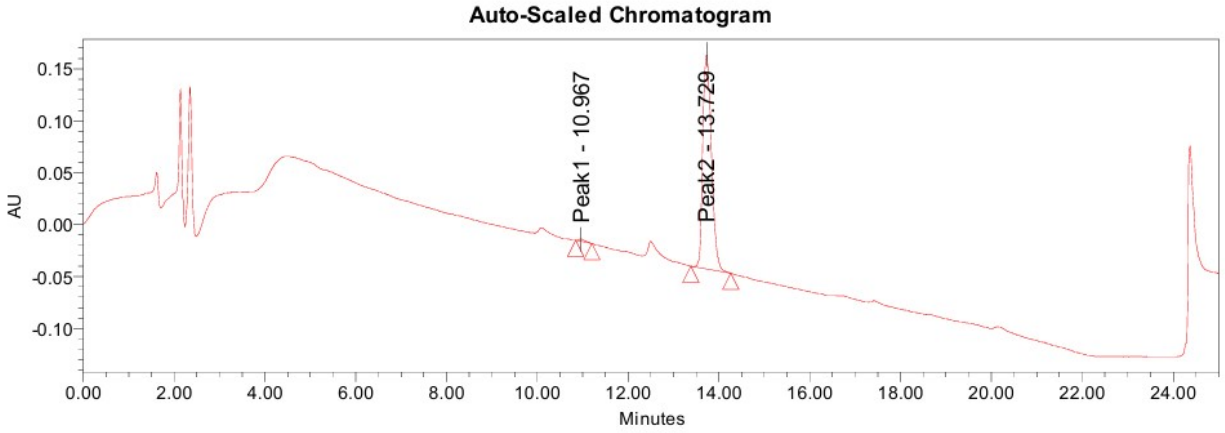
	Name	RT	Area	Height	% Area
1	Peak1	13.296	2653437	250194	99.14
2	Peak2	17.411	23060	2378	0.86

Figure S22. HPLC spectrum of Compound **6b**.

SAMPLE INFORMATION

Sample Name:	TIRZ/25-39- 8 MER ON-C	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	85	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	8 Mer ON_C
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 14:14:35 IST		
Date Processed:	14-11-2025 16:34:45 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5



Peak Results

	Name	RT	Area	Height	% Area
1	Peak1	10.967	22822	2197	0.82
2	Peak2	13.729	2750407	206382	99.18

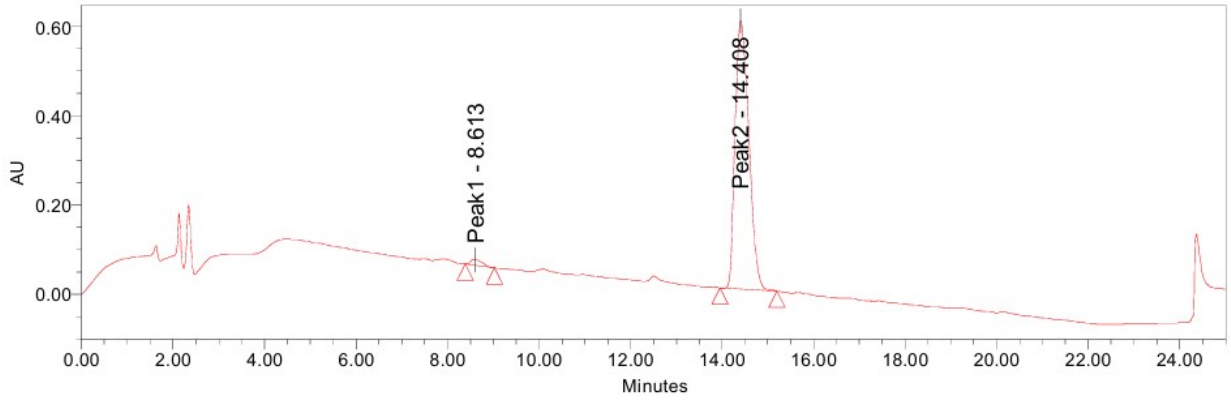
Figure S23. HPLC spectrum of Compound **6c**.

SAMPLE INFORMATION

Sample Name:	TIRZ/25-39- 8 MER ON-D	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	86	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	8 Mer ON_D
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 14:40:34 IST		
Date Processed:	14-11-2025 16:36:26 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5

Auto-Scaled Chromatogram



Peak Results

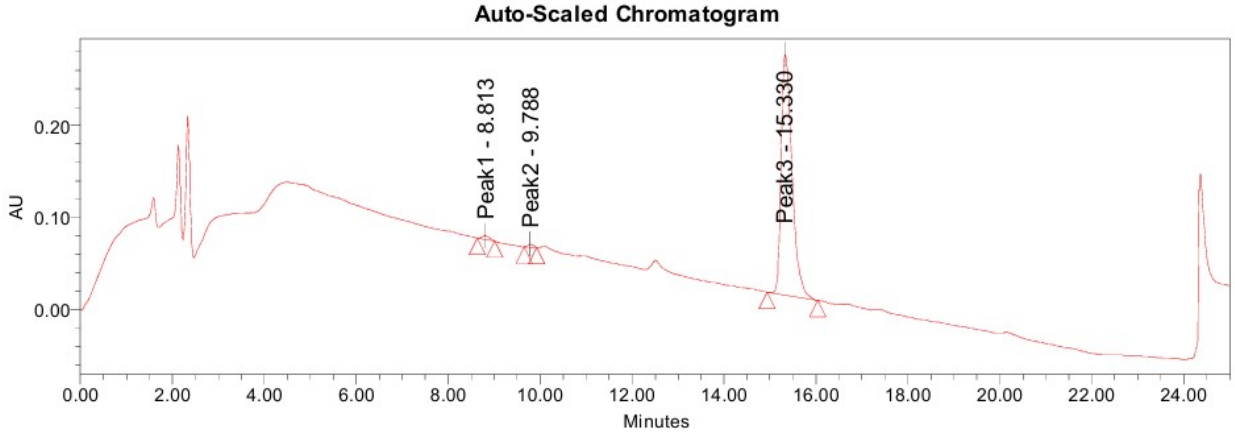
	Name	RT	Area	Height	% Area
1	Peak1	8.613	236006	13190	1.84
2	Peak2	14.408	12561004	601472	98.16

Figure S24. HPLC spectrum of Compound **6d**.

SAMPLE INFORMATION

Sample Name:	TIRZ/25-39- 8 MER ON-E	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	TIRZ
Vial:	87	Acq. Method Set:	SIC_25MI_9505_MS
Injection #:	1	Processing Method:	8 Mer ON_E
Injection Volume:	10.00 ul	Channel Name:	220.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	2998 PDA 220.0 nm (2998
Date Acquired:	14-11-2025 15:06:21 IST		
Date Processed:	14-11-2025 16:38:46 IST		

Buffer Preparation :
Mobile Phase A : 0.1% TFA In Water
Mobile Phase B : 100% ACN
Diluent : Water : Acn (60:40)
Column :ISA Spher100-5 C18 250 X4.6 mm 5 u m
Flow : 1 mL/min
Column Temp: 30°C
Sample Temp :15°C
Gradient Programrme : T/B : 0/5,1/5,15/75,20/100,22/100,23/5,25/5



Peak Results

	Name	RT	Area	Height	% Area
1	Peak1	8.813	45512	4014	1.03
2	Peak2	9.788	28400	3344	0.64
3	Peak3	15.330	4335544	261572	98.32

Figure S25. HPLC spectrum of Compound **6e**.

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